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PAPER CHROMATOGRAPHY

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BY

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of the University of Heidelberg

Second revised and enlarged edition

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LONDON

MACMILLAN & CO LTD

NEW YORK · ST MARTIN'S PRESS

1954

MACMILLAN AND COMPANY LIMITED
London Bombay Calcutta Madras Melbourne

THE MACMILLAN COMPANY OF CANADA LIMITED
Toronto

ST MARTIN'S PRESS INC
New York

PRINTED IN GREAT BRITAIN

FOREWORD

THERE must be very few chemical laboratories today where paper chromatography is not employed. Within only a few years it has become established all over the world. Nevertheless, its growth continues unabated, so much so as to justify this second edition, which is twice the size of the first, published only a year ago. It is no small achievement that, from the formidably large literature on the subject, Dr. Cramer has produced a handbook which is readable, systematic and omits none of the essentials.

Ten years ago, my colleagues, Drs. Martin and Gordon, and I produced our first successful two-dimensional chromatogram. The final stage was carried out in an atmosphere of coal-gas. As the flimsy tin box we used was far from gas-tight, it had to be put outside the laboratory. With its precious cargo, the box was manœuvred on to a narrow window-ledge, situated unpleasantly high over a concrete path, and left there over-night with gas passing in. After this memorable occasion we avoided working on the window-ledge by the simple expedient of blocking the leaks with wet soap, after locating them with a lighted taper passed over the outside of the gas-filled box. Like other established methods, paper chromatography was not brought into the world without considerable birth-pangs, and much could be written about those early adventurous days; perhaps the full story may one day be told—it would make instructive as well as amusing reading.

Although there is much less need nowadays to improvise, because much of the apparatus is readily available, there is still ample scope for adaptation and the exercise of ingenuity with paper chromatography. Many examples will be found in this book, while hardly a week passes without some useful contribution in the scientific press. Dr. Cramer has, by his industry and initiative, produced a book which, I believe, will be a valuable work of reference for a long time to come, and I feel honoured to have been asked by him to write this preface.

R. CONSDEN

EXCERPT FROM THE PREFACE TO THE FIRST EDITION

RESEARCH in the natural sciences is characterized by a well-meditated synthesis of experiment and intellectual conception. Only in experiment can a mental process assume concrete form, and the experiment leads on to new ideas which in their turn give rise to new experiments. Progress in the natural sciences during the last 150 years must thus be regarded as an ever-advancing process of intellectual evolution on the one hand and as a continuous development of experimental technique on the other. These processes are inseparably wedded and mutually conducive in a high degree.

In the course of research undertaken during the last decade, and especially has this been true of biochemical research, it has often been necessary to carry out experiments with minute quantities of substance. The discovery of paper chromatography here constitutes an essential and logical experimental advance which, as has since become evident, has in turn led to a more comprehensive way of viewing problems in the most varied fields of chemical science.

FRIEDRICH CRAMER

Heidelberg, Summer 1951

PREFACE TO THE SECOND EDITION

IN the course of the last year, paper chromatographic technique has once more gained access into numerous laboratories, and it is to be hoped that the first edition of this book rendered much valuable service thereby.

In the present new edition, which has been revised and very considerably enlarged, more attention has been paid to an exact description of experimental methods. The book has also been furnished with several new coloured plates and a transparent key. It is thus hoped that the work will have come closer to its goal of providing a laboratory manual. For this reason it has not been possible to include a comprehensive account of all the work in which paper chromatography has found application. Today its applications are legion, and it would be just as absurd to quote all these in a book intended for laboratory use as it would be to give a complete list of distillations in a handbook on distillation practice. May reference be here made to the meritorious reports on recent advances in ' Analytical

Chemistry' (293). It is safe to assume that the technique employed paper chromatography will gradually become standardized in favour proven methods.

Once again I am indebted to numerous colleagues for valuable sugges tions and advice. I am especially grateful to Herrn Dr. Helmut Zahn fo placing manuscript material at my disposal. My wife rendered assistanc in reading the proofs and compiling the subject index.

FRIEDRICH CRAMER

Chemisches Institut der Universitaet
Heidelberg, Summer 1952

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With 68 figures in the text, 4 coloured plates, 2 transparent keys and 1 folding plate.

PLATES AND KEYS

I. GENERAL SECTION

Historical Introduction

THE practice of chromatographic analysis—viz. the separation of substances by virtue of their differing degrees of adsorption on Al_2O_3 and other adsorbents—has proved to be so extraordinarily useful since its rediscovery in the late '20's that it has now become standard practice in organic laboratories (cf. Zechmeister (361)). Chromatography was originally intended for use solely on coloured substances, but as a result of numerous improvements and modifications it has been possible, especially during the last few years, to adapt the process for employment with colourless materials (45). The Tswett method of chromatography is, nevertheless, limited in its application to the more lipophilic type of compound and just those substances which have come into the foreground of recent research—viz. the fundamental units of proteins and polysaccharides, i.e. the amino-acids and sugars—hardly lend themselves to treatment by this method. Neither has it been possible to discover a simple micro-method, such as that which proved successful in separating α- and β-carotin; and previous methods of separation—e.g. for amino-acids using butanol or precipitation with a specific precipitant—are tiresome and associated with so much loss of yield that satisfactory results could be achieved only by working with relatively large quantities of material. It is therefore hardly surprising to learn that the new method of paper chromatography has its roots in protein research. It was here that the need for an extension of the available experimental methods was most keenly felt. In 1941, '42 and '43 Martin, Gordon and Synge (131) developed a process of partition chromatography for the separation of hydrophilic substances, especially amino-acids. A column of silica gel treated with a definite quantity of water is employed, the acetylated amino-acids being introduced at the upper end. The chromatogram is then developed using one of a whole range of suitable organic solvents—e.g. butanol, petrol ether, chloroform, etc. As a result of differing distribution coefficients of the amino-acids between the aqueous phase (on the gel) and the organic phase, the individual components are washed down at different speeds, thus effecting a separation. This procedure gave excellent results when applied to the structural elucidation of insulin and gramicidin S (269, 270), and really represents the forerunner of paper chromatography. Thus in 1944 Consden, Gordon and Martin (68) hit upon the idea of substituting a simple strip of paper for the column of silica gel. The first results achieved in this manner using free amino-acids were so encouraging that they were immediately acclaimed from all sides and speedily developed. The fact that the English authors chanced to use Whatman No. 1 filter-paper

in their very first experiments must be regarded as a remarkable piece of good fortune, since, as later became apparent, by no means all types of paper are suited to chromatographic analysis.

The traditional method of capillary analysis constitutes a forerunner of paper chromatography (279). In this, a strip of paper or a woollen thread is suspended with its lower end dipping into a dyestuff solution, which solution is then sucked up into the capillary tubes of the material. The dyestuffs thereby become concentrated in definite zones. This process still finds considerable application in the fields of dyeing and colouring, and is only recently beginning to give way to paper chromatography. From a purely physical standpoint, however, this method of capillary analysis is more closely related to adsorption analysis.

Having proved of such immense value in the separation of amino-acids, the paper chromatographic method was soon applied to the analysis of sugars, natural colouring matters, phenols, purines, organic acids, steroids and, more recently, to the investigation of nearly every type of mixture, *inter alia* mixtures containing inorganic components.

The paper chromatographic method is primarily of qualitative application working with quantities in the range 5–50γ of each individual component. Even though numerous successful experiments yielding quantitative results have been carried out, one should never lose sight of the essential limitations of the method or expect too much from it. It is not every problem which can be solved by paper chromatography. However, in co-operation with the methods of classical and modern organic chemistry, it may be regarded as one of the most significant achievements of organic chemical research technique.

General Technique

Before proceeding to a detailed account, let us pause to consider the general method employed in paper chromatographic work and familiarize ourselves with the necessary fundamentals.

The solution to be investigated, which, as a rule, will be an aqueous solution of a hydrolysed protein or polysaccharide, a plant extract or other reaction solution, should be approximately 1% with reference to the substance being estimated. This concentration may, however, be varied within quite a wide range. The solution must be practically free from ions.

Using a micro-pipette, the solution is now applied at a previously marked spot to a strip of filter-paper at least 3 cm. wide and 50 cm. long. The tiny drop then spreads out on the dry paper to a circular spot which is allowed to dry for a short period.

The strip prepared in this manner is suspended so that its upper end hangs over the edge of a trough and dips into the aqueous organic solvent contained therein. The solvent—e.g. water-saturated butanol, aqueous phenol—is now sucked up by the paper and migrates on down past the spot containing the substance under investigation. The individual substances are then drawn along by the solvent at different velocities, depending on the solubility ratio, and thus migrate at different speeds on the paper, just like the zones on a Tswett chromatogram. When the solvent front has almost reached the end of the strip, the latter is removed from the trough and, after marking the position of the front, is allowed to dry. The time required to run 40 cm. varies with the nature of the solvent, but usually amounts to 12–24 hr. Thus it is convenient to commence a run in the evening, so that it is finished by the following morning.

The ascending method is still easier. In this case the paper strip is merely hung up with its end dipping down into the solvent. This is then sucked up by the paper, and carries the substance being investigated at a definite velocity on with it. Ascending chromatograms generally run in ½–6 hr.

It is now necessary to spray the dried chromatogram, which has been treated in the above manner, with some suitable reagent, so as to produce colour reactions in the spots indicating the individual substances. Thus an amino-acid chromatogram would be sprayed with a ninhydrin solution. A chromatogram of this type is shown in Fig. 1. If the substances being investigated are coloured in the first place, then this additional treatment is, of course, rendered unnecessary.

Every compound capable of being analysed chromatographically possesses a definite speed of migration in a defined solvent. Thus long-chain amino-acids migrate more rapidly than glycocoll, monosaccharides quicker than disaccharides, aglycones faster than glucosides, etc. A substance may thus be characterized by the speed at which it migrates.

The R_f-values. A measure of the velocity of migration of a compound is the so-called R_f-value, which is defined as the quotient of the distance of the substance from the starting point divided by the distance of the solvent from the starting point.

$$R_f = \frac{\text{Distance—starting point—substance X}}{\text{Distance—starting point—solvent front}}$$

i.e. the ratio with respect to the front, hence the term 'R_f'. Thus in Fig. 1 the R_f-value of substance $A = R_{fA} = \frac{a}{c}$, and of substance $B = R_{fB} = \frac{b}{c}$. The R_f-values are hence always less than or equal to 1 and independent of the length of the strip. Sharp separation is obtained as a rule only for R_f-values less than 0·90.

R_f-values are subject to variations of up to about 10% as a result of temperature and concentration changes, slight inhomogeneity of the paper, and foreign ions and impurities in the solvent. It is therefore frequently advisable and always safer to run a control substance alongside the unknown on a broad paper strip. This then serves to provide an indisputable reference point. This procedure is especially necessary when dealing with a solution consisting of a number of components and possibly still contaminated with inorganic salts.

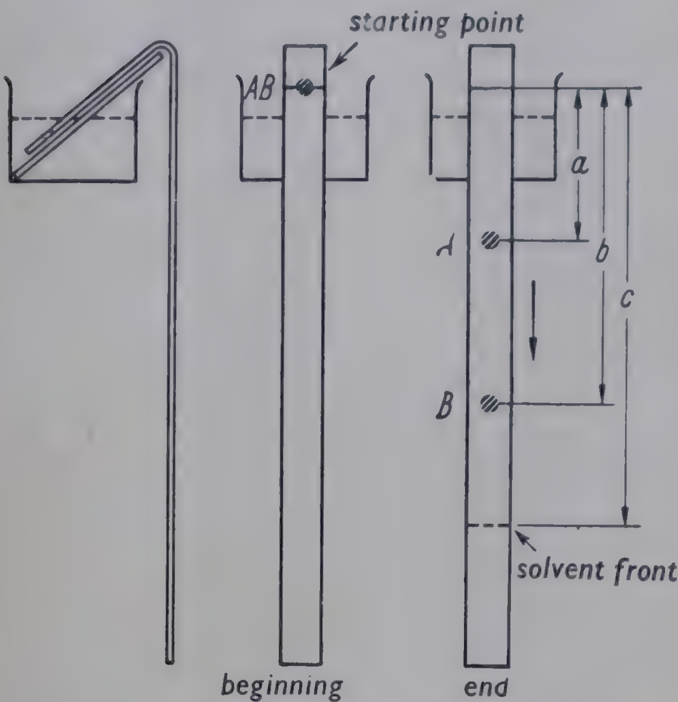


FIG. 1.—Schematic representation of a paper chromatogram.

In order to establish the identity of a substance, this may be chromatographed simultaneously with the suspected pure substance. If the spot then remains uniform in a long chromatogram after being treated with several solvents, the substances may safely be assumed to be identical. In this way it is possible to eliminate the effect of salts or other foreign bodies, since the control substance running together is then subjected to the same disturbing factors. This constitutes the principle of the mixed chromatogram.

The R_f-value of a reference substance is often, especially in work with sugars, arbitrarily given the numerical value 1, thus defining a relative R_x-value. If in our example the reference substance be B, then the R_b-value of substance A =

$$R_b = \frac{\text{Distance of substance } A \text{ from starting point}}{\text{Distance of substance } B \text{ from starting point}}$$

This value is then to a very large degree independent of external experimental conditions. The R_b-value of substance A in Fig. 1 would thus be approximately 0·5, since A has traversed half the distance of B in the same time period. To transform the R_x-value of a substance into its R_f-value, all that is necessary is multiplication by the R_f-value of the reference substance, which may generally be taken from the literature. R_x-values may exceed unity.

$$R_f = R_x \,.\, (R_f \text{ of reference substance})$$

The R_f-value thus represents a simply ascertainable characteristic, in extension of the classical characteristics of melting point, crystal structure,

optical rotation, spectrum, etc. When quoting an R_f-value it is, of course, necessary to state for which solvent it applies, and preferably also the type of paper used.

Theory

1. Tswett chromatography

In order to understand the physical principles underlying paper chromatography, it is first necessary to consider the common Tswett adsorption chromatography (361). In this case some suitable solid material is used to adsorb a dissolved substance out of solution. On developing the chromatogram—i.e. on running through solvent—the adsorbed substance is partially dissolved out, carried on and re-adsorbed. A part of the substance is thus afterwards to be found on the solid adsorbent, the remainder being in the solution. Equilibrium between the adsorbed and dissolved phase is dependent upon the adsorption coefficient of the substance in question, and may be expressed by means of an adsorption isotherm. A substance which is strongly adsorbed naturally migrates slowly, whereas one which is adsorbed to a lesser degree migrates more rapidly. Even when the difference between the respective adsorption isotherms is slight, a separation may still be achieved, since the chromatographic process represents a series of numerous adsorption and elution processes. Mathematically it may be treated like a fractional distillation column possessed of a large number of theoretical diaphragms.

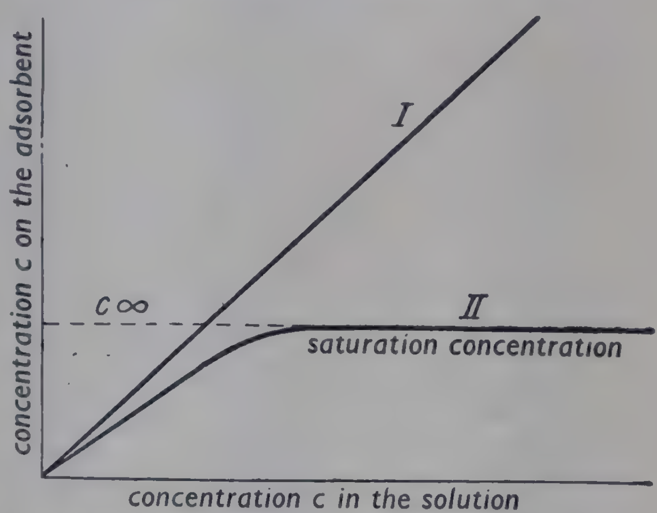


Fig. 2.—Ideal and real adsorption isotherms.

In the ideal case the adsorption isotherm is linear (Fig. 2).

Further conditions for an ideal adsorption are that the equilibrium at each stage should be established immediately, and that no diffusion should occur within the liquid phase. An idealized chromatogram of this type may be represented graphically as in Fig. 3 (210).

A is adsorbed more strongly, and thus has a steeper adsorption isotherm. Assuming that the substances are introduced into the column in equal amounts, the concentration of *A* on the adsorbent will always be higher than

that of B. A therefore migrates more slowly and possesses a lower R_f-valu
In this idealized case the demarcation of the zones is sharp, and there is
variation of concentration within these zones.

Now, in the event of only partial establishment of the adsorption equ
librium or of diffusion of the solutes in the solution—both these deviations gi

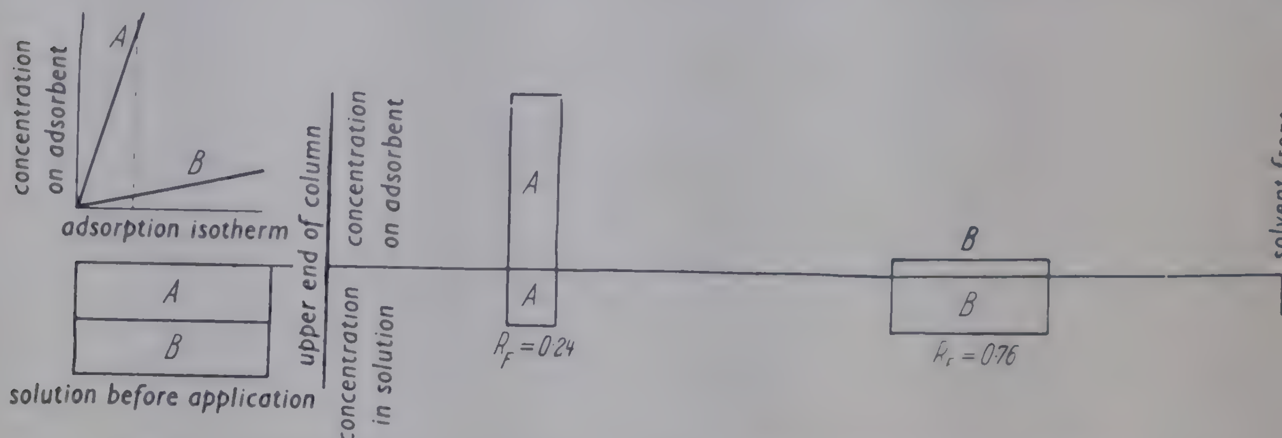


FIG. 3.—Schematic representation of an ideal chromatogram (210).

rise to much the same type of effect—the chromatogram assumes the form depicted in Fig. 4. The bands now have somewhat ill-defined margins although they have retained their symmetry. The same principles, with different assumptions, also apply to paper chromatography. The R_f-values, however, are the same here as in the ideal case.

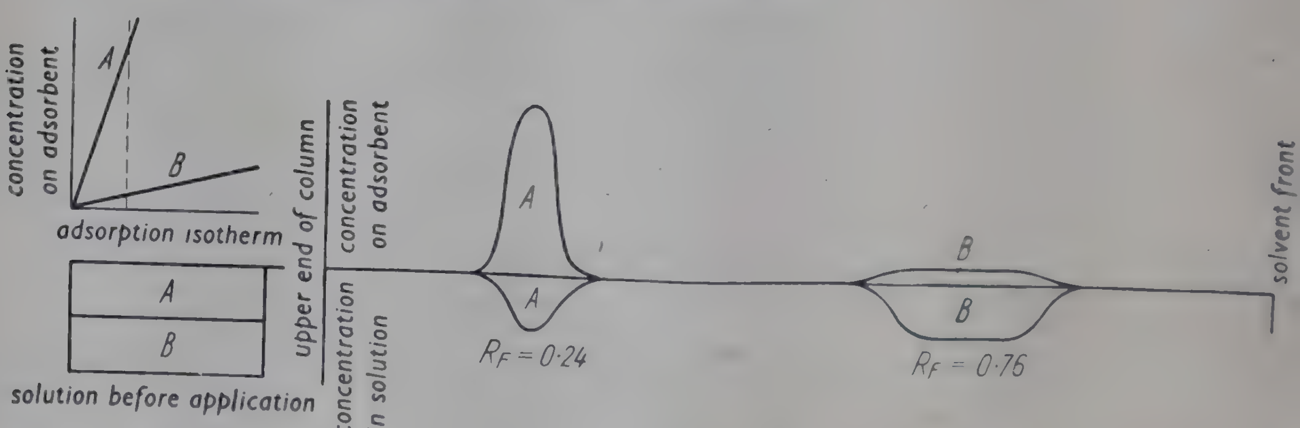


FIG. 4.—Schematic representation of a chromatogram with linear isotherms in which diffusion takes place or in which equilibrium is established slowly (210).

A linear adsorption isotherm signifies that the adsorbent always goes on adsorbing the same fraction of the solute—in the case of A, 75%—and that no saturation of the adsorbing surface ever occurs. In fact, of course, the adsorbing agent is capable of taking up only a limited amount of solute, and the saturation concentration is generally reached even before the adsorbing surface is covered by a layer one molecule thick. The isotherm then follows the curve II (Fig. 2), and as soon as a concentration c has been attained, the surface is exhausted. The course of curve II may be expressed by Lang-

muir's equation. In the case of an adsorption of this type the zones of the chromatogram exhibit protrusions, generally termed tails, which trail behind them. These are characteristic for the Tswett adsorption chromatogram.

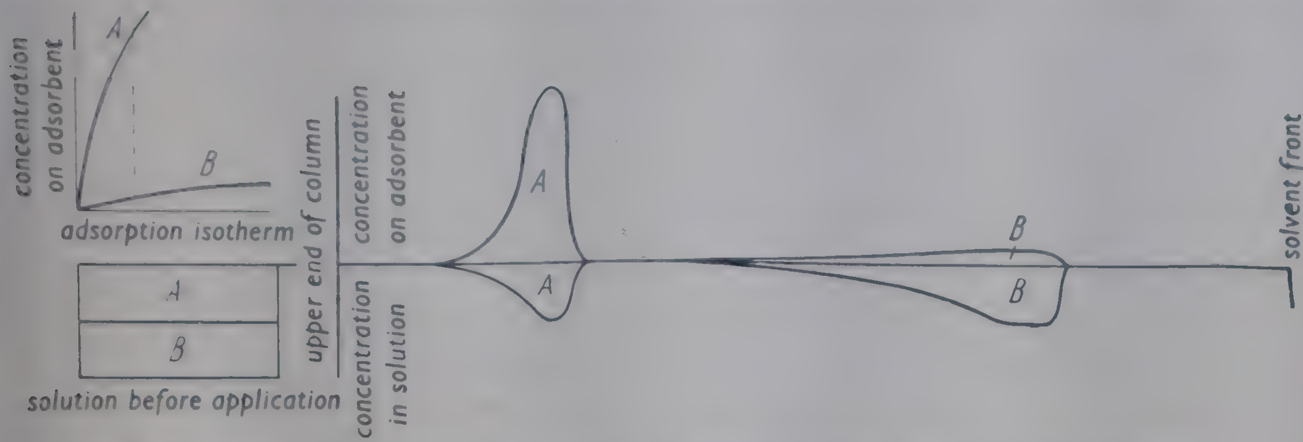


FIG. 5.—Schematic representation of a real adsorption chromatogram (210).

Under the conditions prevailing in Fig. 5, the R_f-values are, of course, greater when the concentration maximum has been passed.

2. Counter-current distribution

Both paper chromatography and adsorption chromatography represent a continuous series of distribution processes. It is possible to follow this continuous distribution process logically through from a single extraction in the separating funnel to the continuous process in a column or on filter-paper.

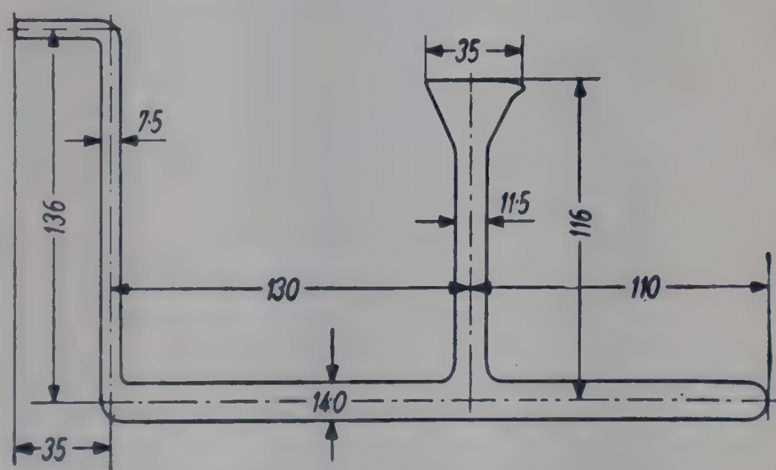


FIG. 6.—Single element of distribution apparatus (319).

The distribution of a substance between two immiscible liquid phases is given by the Nernst equation :

$$\frac{c_1}{c_2} = \alpha = \text{const.}$$

i.e. the distribution isotherm is linear provided neither phase is saturated. The isotherm then has the same form as I in Fig. 2, with the difference that in this case the concentration in the second liquid phase takes the place of the concentration on the adsorbent at the y-axis. As a result of their differing degree of distribution (= the different slopes of the distribution isotherm), two substances become concentrated in different phases of a dual-phase solvent system. The classical method of ether extraction represents a single-stage distribution of this type. Twenty years ago an attempt was made at widening the scope of two-phase

distribution so as to extend it for use as a multi-stage process of fractio separation (168), but the method met with little success, and the mat was dropped.

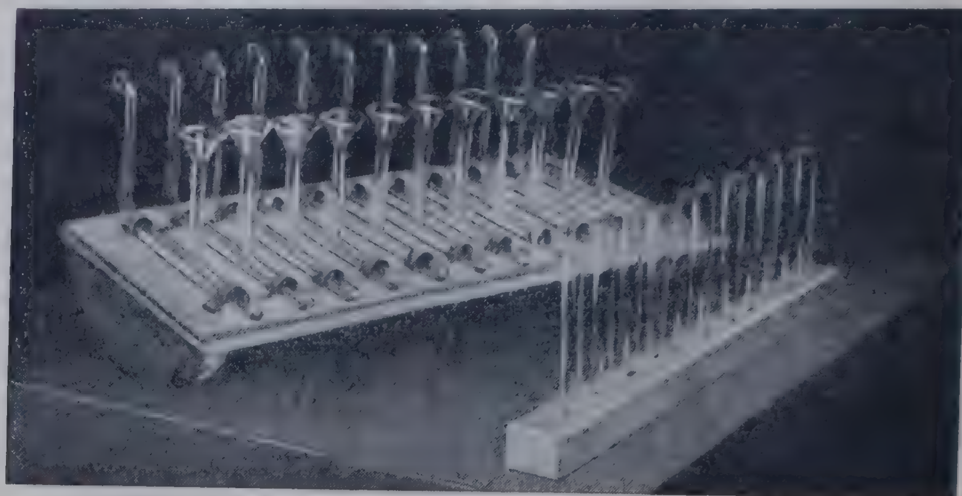


FIG. 7.—Single elements arranged on a frame (319).

In 1941 the problem was again attacked, this time by English worke (209). As already mentioned, they succeeded in separating amino-acid employing a specially constructed distribution apparatus. These worke also focused their attention on the theoretical principles underlying the pro cess. The method was, how ever, first made really practicabl by the work of Craig (74, 75) i this field when it served as method of separation and con centration for a whole series o important natural products Simplified versions of th apparatus suitable for everyday laboratory use have since been constructed by a number o German workers and have proved to be very serviceable (136, 257, 305, 319). A detailed description of one such version is given here as an example (319):

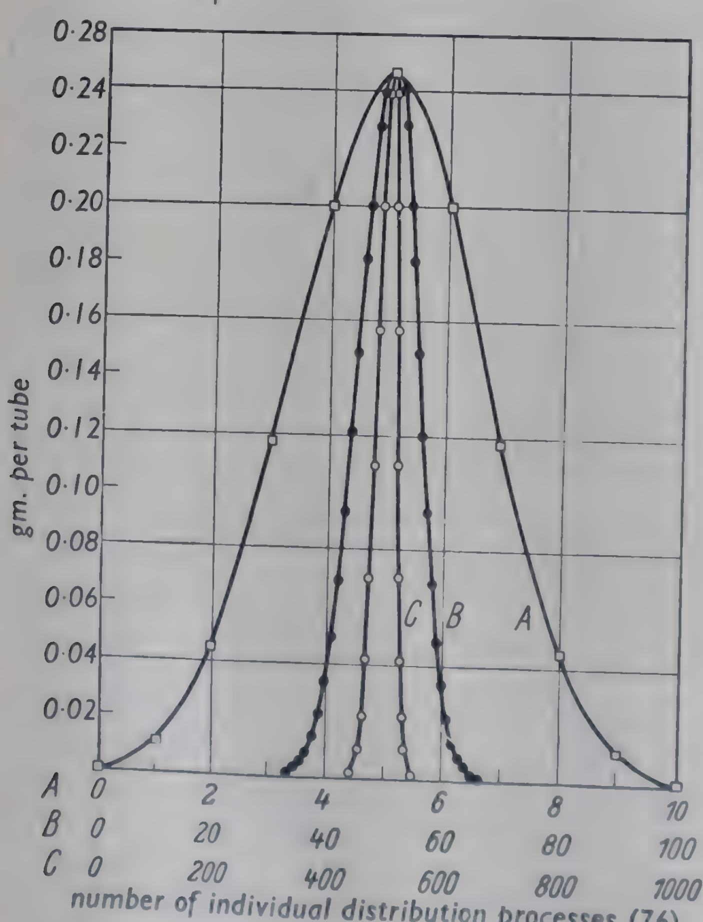


FIG. 8.

The simultaneous transference of all the upper phases is achieved by first tipping them into a second set of vessels, whereupon these are displaced by one unit and then poured back.

The construction of a single element is shown in Fig. 6. For

purposes of filling and emptying out the upper phase, a tube ending in a funnel is attached near the middle. The tube at the left serves to allow the entrance of air when pouring out the upper phase through the central tube and also for tipping out the entire contents at the end of operations. Test-tubes widened at their upper ends and affixed into a suitably bored strip of wood with the aid of paraffin wax are employed for the temporary reception of the upper phases. If it is intended to employ more than one set of elements in series, the last test-tube on the first frame should be supported with the aid of a clamp, since it is necessary to detach it when transferring the upper phase into the first element in the second frame.

Procedure : Assuming the mixture to be separated is contained in the upper phase, all the elements on a frame are filled with equal volumes of the lower phase. This is

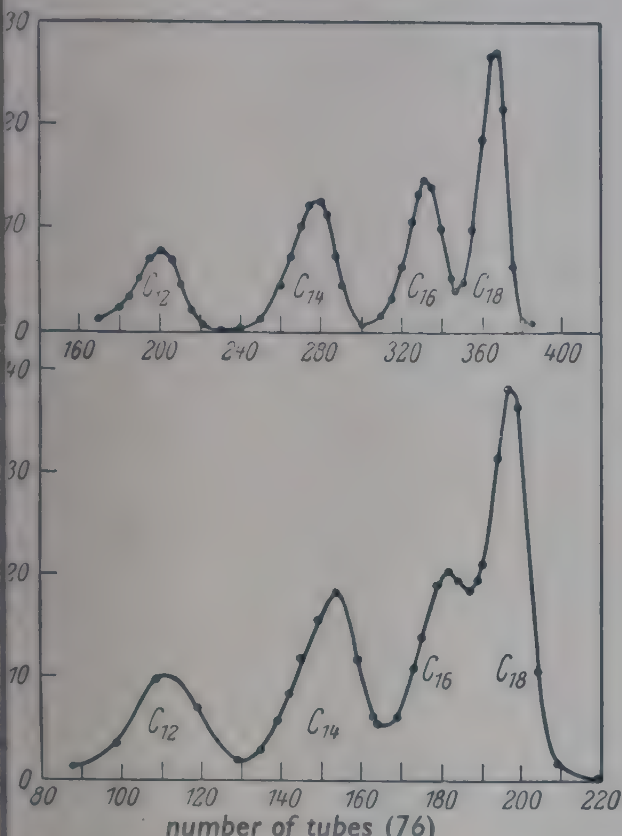


FIG. 9.

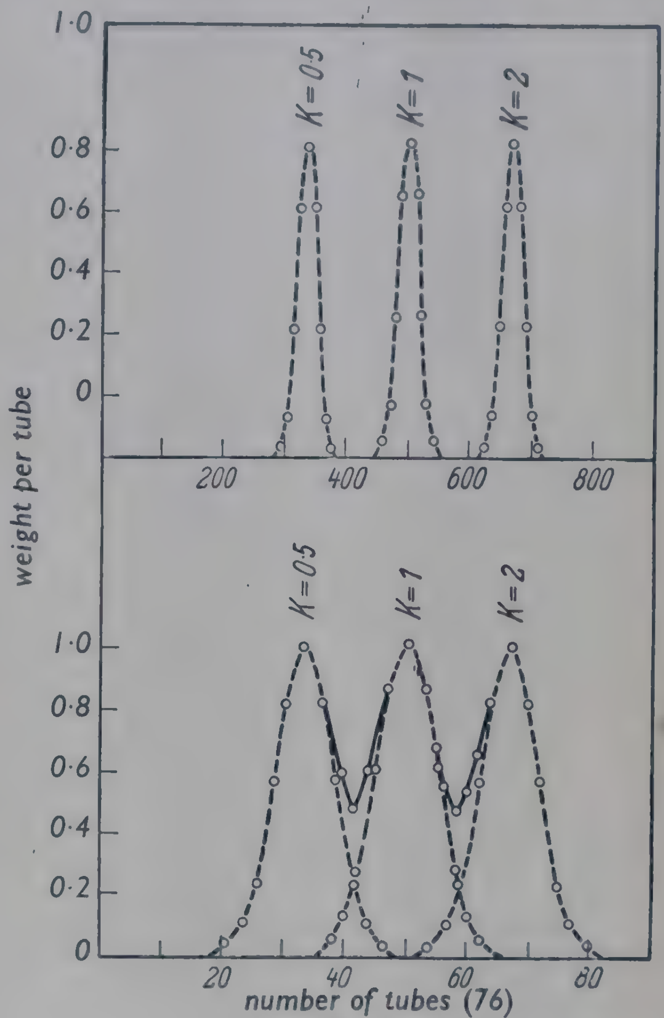


FIG. 10.

achieved by pouring an excess into each element and then levelling off by tipping out through the middle tube. The mixture to be separated, dissolved in the upper phase, is then introduced into the first element and the frame is rocked 20–25 times. The frame is now supported in a slanting position to allow the phases to separate out, after which the first upper phase is poured out into its test-tube. The test-tube rack is displaced by one unit and the first upper phase is tipped back into the second element. Fresh upper phase is now introduced into the first element. After rocking and standing to allow the upper phases to float to the top, the first upper phase is poured off into the first and the second into the second test-tube. In this manner the upper phases are moved on one stage at a time and the individual substances are carried on down the line at different rates. After distribution has been carried out in all the elements in turn, both phases may be poured out simultaneously through the tubes at the rear. It is found most convenient to pour them into a second set of large test-tubes. In a contrivance of this type it is possible to work up amounts from 2–5 gm.

The theoretical treatment of the problem of fractional or counter-curren distribution as put forward by Martin and Synge (209) is closely related to th theory of fractional distillation. The more readily soluble a substance is i the organic phase, the faster it migrates on down the line. If, for example the substance possesses equal solubility in both phases, then its R_f-value i 0·5. The larger the number of distribution processes—i.e. the more tube there are in the line—the narrower the bands and the sharper the separation Craig (76) reaches the same conclusion by rather a different route. Fig. 8 shows the distribution curve of a substance for which $\alpha = 1$, $R_f = 0·5$ employing 10, 100 and 1,000 stages. Fig. 10 demonstrates a similar case fo a three-component mixture and Fig. 9 the separation of four fatty acids using 200 and 400 distribution processes respectively. The fact that the substances are separated much more rapidly as the number of stages increases is particularly clear in this figure.

3. Partition chromatography

Martin and Synge hit upon the excellent idea of carrying out the fractional distribution in a column which would serve as the carrier of a stationary phase. They employed aqueous silica gel and as organic solvents wet chloro-

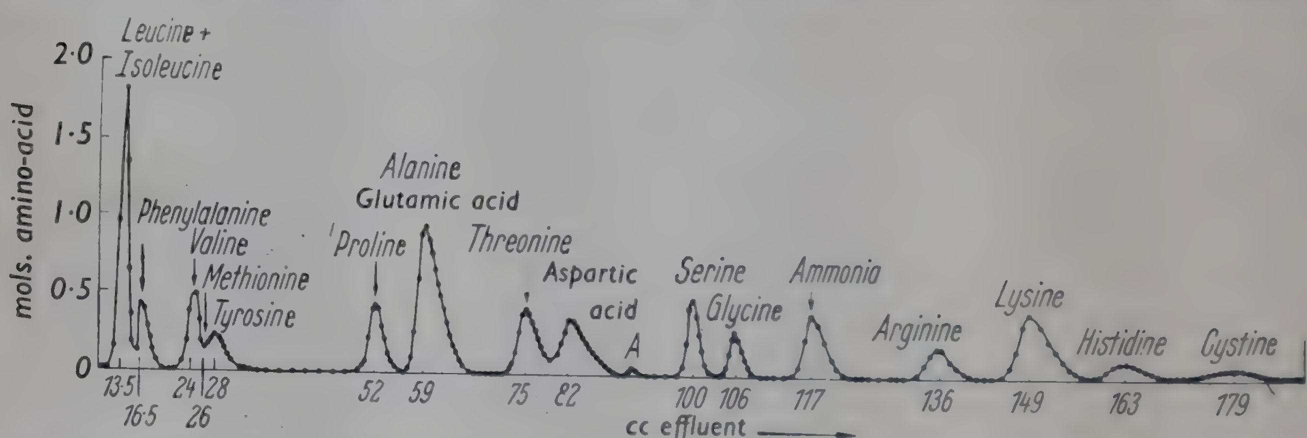


FIG. 11.—Separation of eighteen amino-acids on a starch column. The individual fractions were collected separately at the end of the column (224).

form, chloroform-butanol, propenol-*cyclo*hexane, ether-ligroin, etc. In this way they were able to effect a separation of acetylamino-acids and amino-acids for which an exact pre-calculation of the R_f-values was possible on theoretical grounds. The end-groups of insulin (269), gramicidin (270) and hæmoglobin (254) were estimated in this way.

The preparation of a suitable specimen of silica gel presents no mean difficulty, and must be carried out under carefully prescribed and controlled conditions. The fact that the state of the gel plays such an important rôle, immediately suggests that partition chromatography is not solely based upon a mere distribution process, otherwise the R_f-values would be largely independent of the phase carrier. It is clear that adsorption effects are also at

work here. It is proposed to discuss this problem in a more detailed fashion below. In addition to silica gel, starch has also proved suitable as a carrier for the stationary phase and, indeed, has shown itself superior in many cases (289).

4. Paper chromatography

(a) *Considered as a simple case of partition chromatography*

In its essentials paper chromatography is the same as partition chromatography, the only difference being that strips of cellulose—i.e. paper—are employed in place of silica gel or starch. The damp cellulose constitutes the stationary phase, along which moves the water-immiscible solvent. Considered in this way, paper chromatography is really nothing more than partition chromatography with a linear distribution isotherm, provided the concentration is chosen so that the aqueous phase remains unsaturated. A paper chromatogram should accordingly assume the ideal chromatographic form as in Figs. 3 and 4. The spots of chromatographed substance should neither spread out very much nor should they trail tails along behind them. This state of affairs corresponds to that actually encountered in practice, results similar to Figs. 3 and 4 being obtained. In this diagram it is necessary, of course, to substitute 'distribution' in place of 'adsorption' and 'stationary phase' for 'adsorbent'.

The calculations made for partition chromatography on a silica-gel column may also be applied to paper chromatography. The distribution coefficients α measured directly on the column and the values obtained from the R_f-values on the paper chromatogram, are in good agreement (68).

The connection between α and R_f is given by the formula $\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_f} - 1\right)$, where A_L and A_S are constants of the paper and may be ascertained from experiments with substances of known α and R_f.

	Found	Calculated
Glycocoll . .	70·4	70·4
Alanine . .	39·9	42·3
Valine . . .	10·5	9·5
Leucine . .	5·4	5·5

The concept of paper chromatography as a continuous process of fractional distribution is thus based upon

1. Linear adsorption isotherm.
2. Pre-calculable R_f-values.
3. The analogy between counter-current distribution and paper chromatography.

(b) *An adsorption process?*

During the last few years paper chromatography has also proved to be possible with single-phase solvent mixtures (19, 85, 164, 223, 243). Practically all water-miscible alcohols, organic acids and bases, or mixtures of these, can, in the presence of water, be used as solvents for paper chromatographic work. The results obtained in this manner are quite as good as those using two phases, the separation being equally sharp and the spots round or oval. This would certainly appear to be contradictory to the conception of paper chromatography as a continuous two-phase distribution, which latter view has therefore been contested (76, 84). Craig is of the opinion that the difference between counter-current distribution and partition chromatography is greater than that between Tswett chromatography and partition chromatography. Other authors (84) regard paper chromatography as a case of pure adsorption chromatography, and explain away the linearity of the isotherms by the fact that as a result of the spreading out of the substance on to the relatively large area of a spot, the saturation concentration of the adsorbent is never attained, and so adsorption can occur only in the linear portion of the Langmuir isotherm. In my opinion this explanation is insufficient to account for the fact that, for example, both 100γ and say 0·2γ alanine give, within the limits of experimental error, the same R_f-value on a paper chromatogram (84).

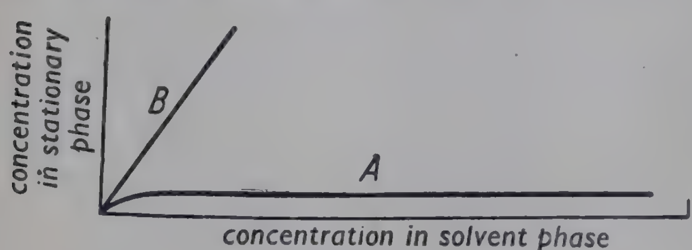


FIG. 12.

It is noteworthy, and of extreme importance for an elucidation of the process of paper chromatography, that a minimum 5% (methanol)–40% (picoline) of added water is an essential constituent of the single-phase solvents used. In the absence of water, very long tails are observed, and in many cases completely useless results are obtained. This signifies that in the absence of water the adsorption isotherm assumes the form *A* in Fig. 12—i.e. the paper fails to adsorb unless water is present. As soon as water is introduced into the system, however, the process again functions smoothly, in accordance with the scheme shown in Figs. 3 and 4; the normal distribution isotherm *B* takes the place of the isotherm *A*.

The cellulose structure of the paper used in chromatography is characterized by the presence of small areas of crystalline polysaccharide which have a strong tendency to swell in water. The same applies to the starch used in partition chromatography, and a similar state of affairs is also present in silica gel. The cellulose and starch are thus able to trap considerable quantities of water within their lattice structures, and this water can, by virtue of the numerous OH-groups of the polysaccharide, be bound very tightly. One thus has to imagine the cellulose taking up the 5%, or more, of water

in the one-phase solvent and being transformed into a swollen state, which then represents the chromatographically active phase. In crude terms, the cellulose surrounds itself with a hydrate sheath, which then acts as the aqueous phase in the partition chromatogram. The hydrophilic molecules thus dissolve in this phase, where they remain firmly attached and establish equilibrium with the aqueous organic phase streaming past. Even with a single-phase solvent it is thus still possible to speak in terms of a distribution—i.e. the distribution between an aqueous phase—consisting of the water in the solvent and on the cellulose, and an aqueous organic phase which flows past the water-sheathed cellulose. The separation into two phases is thus first effected by the water-attracting properties of the cellulose. A certain measure of adsorption is naturally to be expected, especially with substances inclined to form hydrogen bonds, but, nevertheless, in its essentials, the process is still one of partition or distribution. The above interpretation satisfactorily explains the similarities, and also the occasional discrepancies, between paper chromatography and fractional distribution (298, 194, 213).

Scope

Since paper chromatography is essentially a distribution process between a stationary aqueous and a moving organic phase, the substances to be separated must be at least slightly water-soluble, otherwise they will not be retained at all by the damp paper and will not then, of course, form any discrete spots. As a general rule, no useful results are obtainable for a substance migrating with an R_f-value greater than 0·8. Insufficient attention is frequently paid to this fact. It should always be remembered that as symmetrical a distribution in the two phases as possible is a pre-condition for every fractionation effect. This is just as valid for distillation as for chromatography: a vapour which distils over quantitatively as soon as the column is warmed cannot be sharply fractionated. It may also be seen from Figs. 3 and 4 that the spot made by a substance grows larger, the greater the R_f-value. On the other hand, the substance in question must possess at least a slight solubility in the organic phase, otherwise the spot will simply fail to migrate at all. In the case of extremely hydrophilic and consequently slowly migrating substances, it is frequently of help to continue running after the solvent front has reached the lower end of the strip. The organic phase is allowed to run and drip off at the bottom for several days (continuous-running chromatogram). Of course, under these conditions it is not possible to evaluate the R_f-value directly; this can only be inferred from a known compound running simultaneously (R_x-value). Substances with low R_f-

values generally give particularly well-defined spots, even when run for long periods.

In order to be in a position to separate the more strongly lipophilic substances, reversed phase paper chromatography has been developed. For this the paper is pre-treated *inter alia* with rubber latex (24, 33, 163, 241). In this way the less polar organic phase is rendered stationary and the aqueous phase migrates. Substances originally having R_f-values which are too large, then appear with small and serviceable R_f-values.

Paper chromatography is only applicable provided that the substances to be separated can be made visible on the paper. Since, however, colour reactions are either already known or, at all events, could readily be developed for practically all organic substances, this condition places little limitation on the scope of the method. Nevertheless it is generally found necessary to modify the standard colour reactions (ninhydrin, silver nitrate, ferric chloride, etc.) to suit the special conditions prevailing on filter-paper. After standardization, reproducible and specific results may be achieved. For a more detailed discussion of this aspect, the reader is referred to the special section. Fluorescent substances may, of course, be located directly under a suitable lamp without previous treatment. Radio-active material can be detected in minute traces radiographically.

It is thus possible to separate and characterize a substance on the paper chromatogram if

(1) it is at least slightly soluble both in water and in the organic phase;
(2) it can be made visible on the paper by some physical property or chemical reaction;
(3) it reacts neither with cellulose nor with the solvent employed.

The following list presents a survey of the classes of compounds which have hitherto been successfully separated:

Amino-acids	Steroids
Peptides	Phenols
Proteins	Antibiotics
Sugars	Higher alcohols
Purines, nucleic acids	Technical dyes
Organic acids	Alkaloids
Anthocyanins	Pterines
Flavones	Inorganic ions

Experimental Technique

1. Sensitivity

The paper chromatographic method may normally be applied to quantities ranging up to a maximum of 60γ for each component. Thus, supposing a mixture of ten amino-acids is to be separated, the total amount of substance chromatographed should not exceed 600γ. If larger quantities are employed, then the polar phase is not in a position to dissolve all the material and so it is impossible for a genuine state of equilibrium to be established, with the result that the spots obtained are no longer well-defined. Sugars, however, may be applied in somewhat larger quantities.

The lower limit is conditioned solely by the sensitivity of the colour

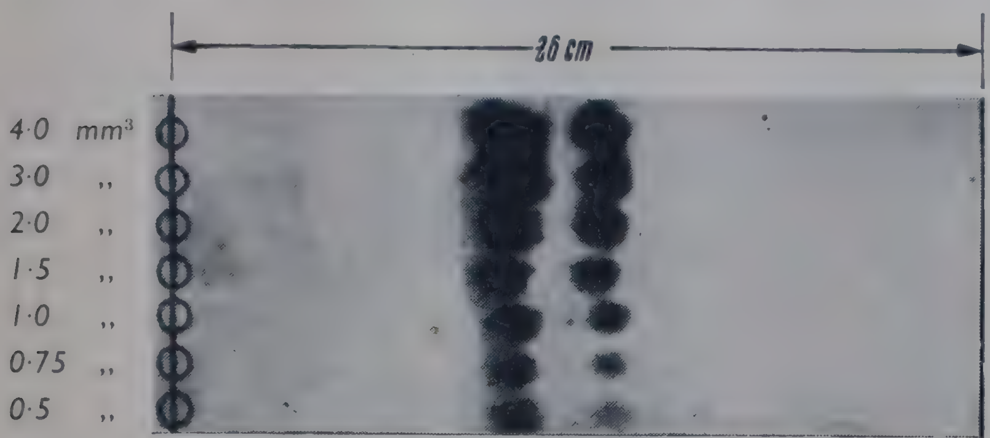


FIG. 13.—Paper chromatogram of a 1% solution of glucose and xylose which was applied in continuously decreasing quantity (237).

$40\gamma = 4{\cdot}0$ mm^3	$15\gamma = 1{\cdot}5$ mm^3
$30\gamma = 3{\cdot}0$,,	$10\gamma = 1{\cdot}0$,,
$20\gamma = 2{\cdot}0$,,	$7{\cdot}5\gamma = 0{\cdot}75$,,

$5\gamma = 0{\cdot}5$ mm^3

reaction. Thus the presence of as little as a single γ of amino-acid may be demonstrated quite comfortably with the ninhydrin reaction. The lower limit of sensitivity for sugars lies between 1 and 2γ.

The greater the amount of substance applied, the larger the spot obtained on the chromatogram and the higher the degree of uncertainty in the R_f-values. It is therefore expedient to run the smallest amount of substance consistent with obtaining good, clear spots—i.e. 10–30γ of each component.

Attention is here expressly drawn to the tendency shown by beginners in this field to apply amounts of substance which are nearly always too large. Before commencing, it is essential to work out the rough concentration of the solution being used or to make preliminary experiments with a view to ascertaining the most suitable concentration—and then to proceed accordingly. Paper chromatography is a thorough-going micro-method, and those unaccustomed to working on this scale are very prone to the errors of the novice.

If one component is present at a much lower concentration than the remaining ones—say 1 : 200—then a preliminary enrichment of the solution with respect to this component is necessary, since it would not be possible to apply as much of the original mixture to the paper as would permit of a recognizable colour reaction. The method employed for this preliminary concentration must, of necessity, depend on the nature of the substances present, and thus no definite rules can be given.

2. The spotting solution

This should be approximately 1% with respect to each component. Two mm.[3] of the solution must then be applied assuming 20γ are to be chromatographed, and this volume should then spread out to a spot of about 1 cm. in diameter. A higher concentration than this makes the initial spot too concentrated (tail formation), whereas for a lower one, the spot would need to be too large.

Should the given solution be too dilute, it may be concentrated on the paper itself by applying several batches at regular intervals, leaving sufficient time for evaporation between each application. A suitable arrangement for this purpose is cited below (307).

When working quantitatively with a few c.c., it is especially irksome to have to evaporate down before spotting. A combination of concentration and application is best achieved as follows : The solution is gradually allowed to drop from a fine pipette on to the paper while simultaneously blowing on a draught of hot air.

The patent hot-air dryers, such as those used for drying films in photography, may be conveniently employed for this purpose. The speed of application should naturally be adjusted to suit the speed of drying. The wet spot should not exceed 1·5 cm. in diameter.

The original solution need not necessarily be aqueous, since the solvent is, in any case, evaporated away before commencing a run. Acetone, benzene, alcohol, etc., may thus also be used, and possess the advantage of drying more rapidly. However, it is generally aqueous solutions of amino-acids, sugars, etc., which come into question.

3. Interference due to foreign material

The components of a mixture being separated exert no appreciable mutual influence as long as the concentration chosen is not too high. Thus the R_f-values remain constant in mixtures too. Inorganic salts and ions may, however, if they are present in fairly high concentration, lead to considerable variance in R_f-values and also to tail formation.

This is due to the fact that ions act as hydrophilic particles and attract water, thus upsetting the equilibrium of the water between the phases. It should be noted in passing that anions and cations migrate at different velo-

cities (cf. section on inorganic paper chromatography). The following methods may be employed for removing inorganic material :

(*a*) *Precipitation* : e.g. the solution resulting from hydrolysis of a polysaccharide, containing sulphuric acid, may be precipitated with $Ba(OH)_2$ and filtered in the normal manner. After this treatment it should be sufficiently free from ions.

(*b*) *Ionic exchange* : inorganic acids are retained by basic ion exchangers like 'Wofatit M (W.Z.)'. 'Zeo-Karb 215 (W.Z.)' and other substances. Inorganic salts in neutral solution may be split and the cations retained using 'Wofatit' or some other suitable material. The solution is then acidic when it flows out of the exchanger, and after treatment with 'Wofatit M' is free from ions altogether.

The German 'Lewatits' (Bayerwerke Leverkusen) and the American 'Amberlits' (Roehm & Haas Co. Philadelphia) and 'Dowex' ion exchangers at present offer a wide range of suitable ion exchangers in all grades of acidity.

(*c*) *Electrodialysis* : amino-acids may be largely freed from ions by electrodialysis. A simple apparatus specially constructed for this purpose is shown in Fig. 14 (86, 130).

(*d*) Sugar solutions may be freed from ions by treatment with pyridine, which takes up the sugar, leaving inorganic salts behind (204).

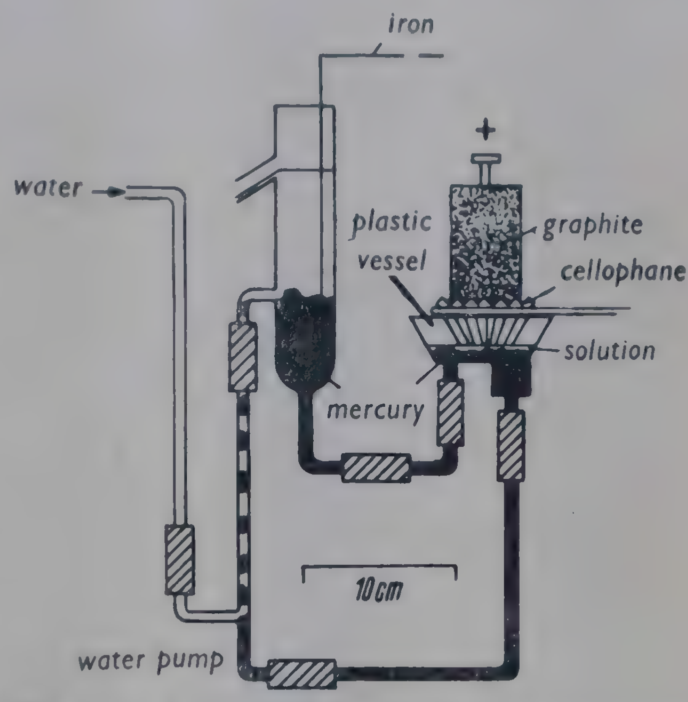


FIG. 14.—Apparatus for removing salts from solutions of amino-acids by electrodialysis (130).

4. The paper

The paper must be of the pure linters type containing no soluble (also in organic solvents) sizing or other additions. It should possess quite definite absorbent properties and an extremely even texture.

The English 'Whatman No. 1' (Reeve Angel & Co., Ltd., Bridewell Place, London, E.C.4) fulfils these conditions ideally.

'Whatman No. 1' was the first paper to be used chromatographically, and for this reason most of the R_f-values in the literature refer to this paper. Other manufacturers have since taken up production of similar types of paper—e.g. Schleicher & Schuell.

Whatman No. 1.	Standard slow-running paper.
Whatman No. 4.	Faster running—for some purposes too rapid. Good for ascending chromatograms.
Whatman No. 3MM.	A thick paper for quantitative chromatograms.

Whatman No. 54.	This paper resists tearing even when wet, and is therefore especially suitable for long chromatograms. It has proved of particular value as carrier for aluminium oxide and calcium carbonate when the use of an impregnated paper is necessary.
Whatman Cellulose Powder.	For chromatography on cellulose columns. Available in two grades, 'Ashless' and 'B'. One or other of these powders will be found suitable for all procedures requiring the use of a column.

In addition to these original types, a number of equally suitable varieties have subsequently been developed in Germany.

Schleicher & Schuell 2043b.	A pure linters paper having approximately the same properties as Whatman No. 1. It has proved suitable for practically all separations and is now in fairly general use in Germany. Weight per sq.m. 120 g. Since all papers run more quickly in the direction in which they have been machined, the S. & S. 2043 is either marked with an arrow or is 2 cm. longer (58 × 60) in this direction.
Schleicher & Schuell, 2043a.	As above. Weight per sq.m. 80 g.
Schleicher & Schuell 598G.	A soft, fast-running paper suitable for preliminary test experiments.
Schleicher & Schuell 2045a and b.	Very high-quality linters papers which run considerably slower than 2043. Separation is very sharp. Also provided with an arrow as 2043.
Schleicher & Schuell 2045a and b.	Fast-running. Correspond approx. to Whatman No. 4.

A few cellulose papers which have proved useful in individual cases are enumerated below.

Schleicher & Schuell.	No. 1101 and 1101L.
	,, 1102 and 1102L.
	,, 1104 and 1104L.
Schleicher & Schuell 1507.	Suitable for sugars, but not for amino-acids (320).
Mahery & Nagel No. 214.	Has yielded noteworthy results with fats (172).

American varieties (184). The suitability of American papers for chromatographic purposes, with special reference to the amino-acids, is given in the following table, in which the papers are arranged in order of their serviceability with various solvent mixtures.

Solvent used	Type of paper				
Collidine	W 3,	SS 595,	W 4,	W 1	
Phenol	W 3,	SS 595,	W 1,	W 4	
Butanol	SS 589,	Black ribbon,	SS 595,	SS 598,	W 3
Butanol—formic acid	W 3,	W 1,	W 2,	SS 602	
Butanol NH_3	SS 589,	Blue,	— Red,	—White,	W 1

W = *Whatman* (Engl.), SS = *Schleicher & Schüll* (Americ.)

'Eaton and Dikeman, No. 613' paper is very versatile.

5. Solvents

These must be at least partially miscible with water. A high vapour pressure gives rise to inconvenience in handling. As a rule, previous purification is necessary. The most common are:

(*a*) Phenol. Pure phenol is shaken with water in a separating funnel, after which the organic phase, which has become liquid, is separated off for use. Purification is necessary (96, 237, 332):

Liquified phenol is distilled over 0·1% Al-turnings and 0·05% sodium bicarbonate at normal pressure until all the water has passed over and then at 25 mm. Hg until only about 20 c.c. of an almost black residue remain. The phenol may also be shaken for several days with 1% ammonia and subsequently purified by steam distillation. Vacuum distillation over zinc dust also yields a sufficiently pure product.

Phenol gives high R_f-values. It is particularly suitable for sugars and amino-acids.

Addition of HCN to the phenol inhibits autoxidation. By working in an atmosphere of H_2S, the disturbing effects of any traces of heavy metals on amino-acids are eliminated, since such metals are bound by the H_2S. Since both gases are present in traces in ordinary coal gas, it is convenient to chromatograph in an atmosphere of coal gas.

(*b*) Butanol in various admixtures is the most widely used solvent. Distillation at normal pressure suffices for purification.

(*c*) Butanol mixtures. The R_f-values in butanol by itself are very low. Various mixtures are therefore preferred.

Butanol–acetic acid: 40 c.c. butanol, 10 c.c. glacial acetic acid and 50 c.c. water are thoroughly mixed (237). It should be noted that in three-component systems the quantity of water cannot be arbitrarily chosen, but must always remain fixed.

Butanol and acetic acid esterify after a few weeks, so that mixtures cannot be kept for long periods, Secondary alcohols esterify considerably slower.

Further butanol mixtures are considered in the special section under separation of individual substances.

(*d*) Collidine. The following procedure may be employed for purification (237).

1 lit. *s*-collidine is cautiously shaken with 5–10 c.c. bromine and then filtered. The filtrate is then shaken with technical sodium thiosulphate and again filtered. After standing for 24 hr. over solid caustic soda, the liquid is once more filtered and distilled. B.p. 172° C.

All these solvents represent systems which are saturated with water at room temperature. They are therefore extremely sensitive to small fluctuations in temperature, a slight fall in temperature being sufficient to cause phase separation—i.e. the appearance of water-drops. This temperature effect may be circumvented by treating the solvent, after saturation with water, with a small amount of the pure organic phase. A solvent pre-treated in this manner is then not completely saturated with water, and is able to adjust itself to small temperature changes.

(*e*) Solvents completely miscible with water (19, 85) may also be employe but these must contain a certain small quantity of water, otherwise the su stances either refuse to migrate at all or produce useless blotches. The *F* values in these solvents are dependent to a high degree on the water conte If the water content is raised too much, all the R_f-values will be fou squashed together between 0·85 and 0·95. The following solvents come in consideration here: alcohols, pyridine and homologues, carboxylic aci acetone, tetrahydrofuran.

So-called *s*-collidine, which is a mixture of collidine and lutidine, m conveniently be replaced by a mixture consisting of 60% α-picoline and 40 water,* which mixture also possesses the advantage of being cheaper.

Further solvent mixtures are dealt with in the special section. T possibilities of variation are almost infinite.

Volatile solvents and ternary mixtures, lacking the thermal degree freedom, must be employed under very carefully controlled conditions—i in a thermostat.

If it is desired to maintain a uniform atmosphere, a piece of iron wire m be attached to the end of the paper strip, which is then kept continuous swinging with the aid of a magnet situated outside the apparatus (112).

6. Application of the solution

The solution is best applied to the paper with the aid of a micro-pipet graduated in mm. The pipettes used for taking blood samples are well suit

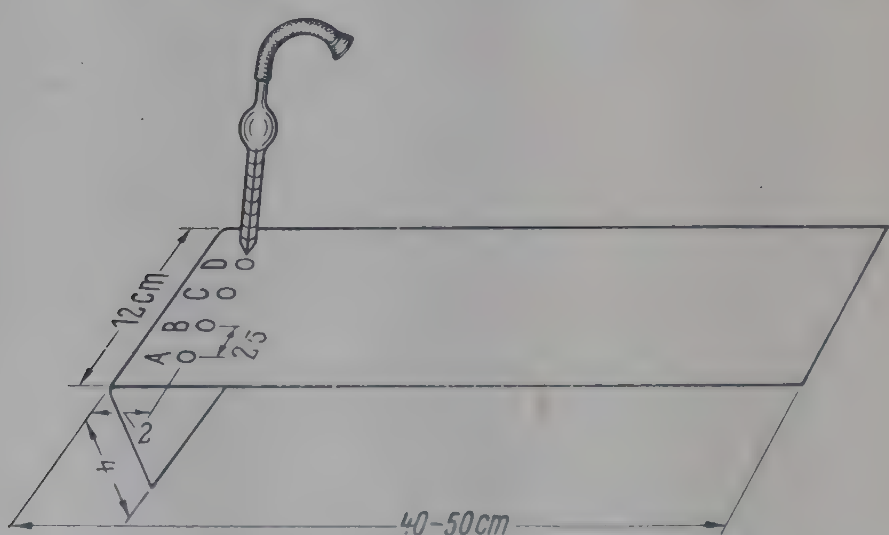


Fig. 15.—Application to the paper.

to this purpose and are also readily available. The graduated lower porti should hold about 4 mm. and may be conveniently calibrated with mercur Of limited application are melting-point tubes with their ends cut off.

* Unless otherwise indicated, figures given for solvent mixtures always refer to parts volume.

After some little practice, drops may also be applied with a glass rod. There is always the danger here, however, of applying too much solution, since 4 mm.^{3} of a 1% solution, corresponding to 40γ, are less than one drop. To begin with, one should therefore work with a fine pipette in order to control the amounts applied with certainty. A micrometer pipette becomes a necessity when working quantitatively. The 'Agla Micrometer Syringe' as supplied by Burroughs Wellcome & Co., London, has proved itself thoroughly satisfactory in this field.

This device has a working capacity of 0·5 c.c. and graduations every 0·2 mm.^{3}, which permit of readings to an accuracy of $\pm$0·05 mm.^{3}

A starting line approximately 6 cm. from the end of the strip is drawn in pencil (not copying pencil !), and the individual starting points for the various solutions being investigated are now arranged at 3 cm. intervals along this line. The solutions are then applied at the starting points by direct contact with the nozzle of the pipette and allowed to soak into the paper (Fig. 15).

The spots dry in about 2 min., after which the strip can be folded back above the starting line and hung in the trough.

7. The trough

Many modifications have been suggested for the trough which serves to hold the solvent. This should preferably be 15 cm. long, 3–4 cm. wide and

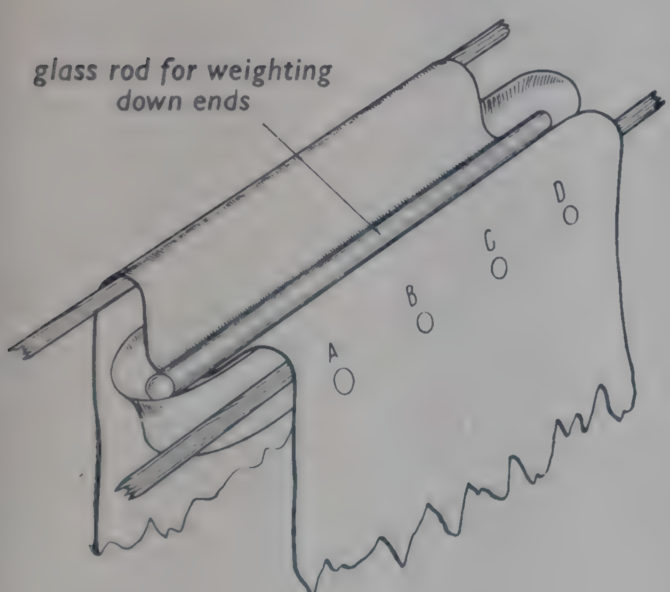


FIG. 16.

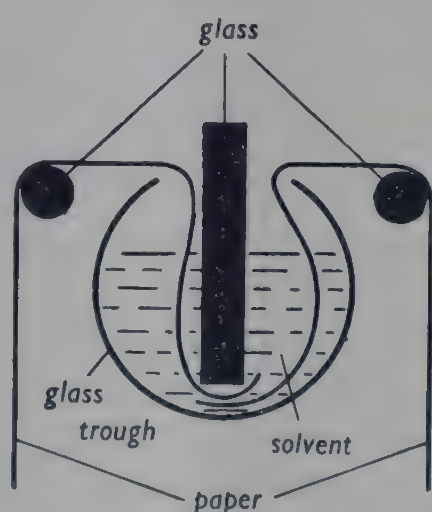


FIG. 17.—Transverse section through trough for one-dimensional chromatogram (68). A trough with V-shaped section may also be employed.

of glass, porcelain, V2A-steel or plastic. A glass rod should be supported above or at the side of the long rim of the trough. The strip of paper can then be hung over this at the fold, with its shorter end dipping down into the

solvent. If the paper were hung directly over the edge of the trough, with a glass rod, the solvent would be sucked up unevenly and also show a tende to flow over. Two chromatograms may be run simultaneously from trough.

8. The complete apparatus

During a run the solvent must not, of course, be allowed to evaporate on the paper. It is therefore necessary to place both strip and trough i closed system which is saturated with vapour from the solvent. Choice o

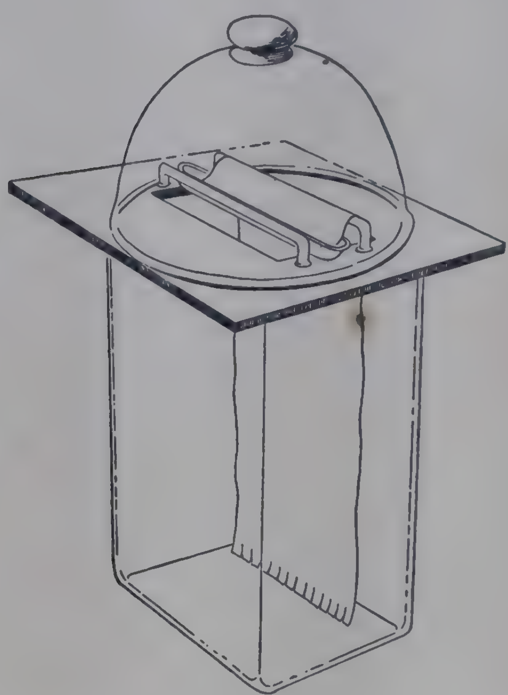


FIG. 18*a*.

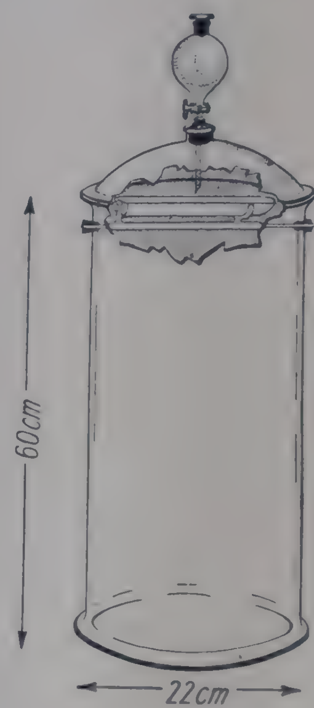


FIG. 18*b*.

FIG. 18*a*.—Apparatus for one-dimensional descending chromatograms (356). · Th lower portion consists of an accumulator vessel. The horizontal plate is of woo having a layer of spongy rubber on both sides, so as to give a gas-tight fit. Th apparatus is closed with a suitable desiccator lid. The trough rests on the woo and the glass rods for holding the paper are let into the wood.

FIG. 18*b*.—An all-glass apparatus as described in the catalogue of Hormuth & Vette Heidelberg, Germany. Fresh solvent may subsequently be run down into th trough without removing the lid. This avoids upsetting the saturation of th atmosphere.

suitable cabinet may, as in the case of the trough, be guided by the materi at hand, but the one chosen should preferably be of glass, or at any rate shoul possess transparent windows. In our laboratory we generally make use o the glass vessels from wet batteries which have ended their term of servic These are about 50 cm. high and may be covered over with bell-jar lids (Fig 18*a*).

The bottom of the cabinet is covered with a layer of the same solvent a in the trough, and this is sucked up on to filter-paper stuck to the side-walls

Complete saturation of the inside atmosphere then results, in consequence of the large surface area exposed.

When working with a variety of solvents, or when it is necessary to make frequent changes of the solvent in the cabinet, the solvent in question may be placed in a dish at the bottom of the cabinet. On suspending the paper it shows a marked tendency to curl up, since it has generally been freshly cut from the roll. The degree to which it hangs vertically is a measure of the amount of vapour it has absorbed from the atmosphere in the cabinet. Provided this atmosphere is sufficiently saturated, the strip should hang vertically within 5–10 min.

After freshly charging a new cabinet, it is necessary to wait at least 24 hr. before commencing a run, to allow the atmosphere inside to become saturated. A number of little incisions must be made at the bottom of the strip, as shown in Fig. 18*a*, when operating a continuous-running chromatogram. This enables the fluid to discharge regularly at the base. A better method is to attach a pack of cotton wool at the bottom (216). This then absorbs the descending solvent. A similar method may be employed with ascending chromatograms.

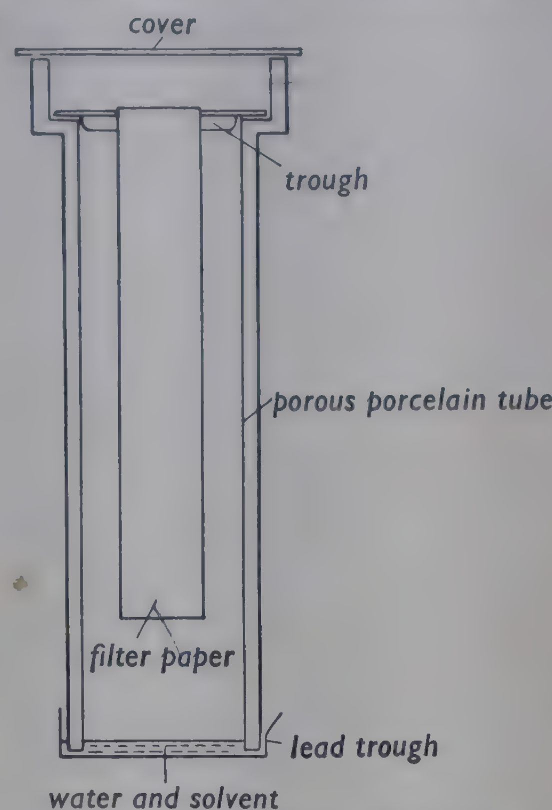


FIG. 19.—The original apparatus of Consden, Gordon and Martin (68).

9. The ascending method

The descending method has been dealt with first here since it was the first to be developed. However, it is the essentially simpler ascending method (339) which should first be considered when investigating unknown substances, and only if this fails to yield the desired results will resort be had to the descending method.

The ascending method (Williams, Kirby) always leads to positive results if there is a fairly large difference in the R_f-values. This is because it is not possible to employ strips much longer than 30 cm. here. Substances having R_f-values differing by 0·03 then appear 1 cm. apart, which is usually just about the lower limit of differentiation. Using a longer descending chromatogram, substances having R_f-values differing by considerably less than this may be separated with ease.

The ascending chromatogram has in addition to its extreme simplicity—it can be carried out quite easily with no more apparatus than a piece of wire,

a paper clip and a clock glass—yet another advantage: a very large number of substances may be run side by side (cf. Fig. 20). The technique is usually as follows:

A sheet of paper of dimensions about 25 × 30 cm. is marked with a starting line 3 cm. from one edge and the solutions are then applied at points along this line. The sheet is curved round to form a cylinder, which is prevented from unrolling by means of a paper clip, and stood in a Petri dish containing solvent. The solvent is thus sucked up and takes care of the separation. Narrower strips may be suspended in any convenient tall vessel, dipping into solvent at the bottom.

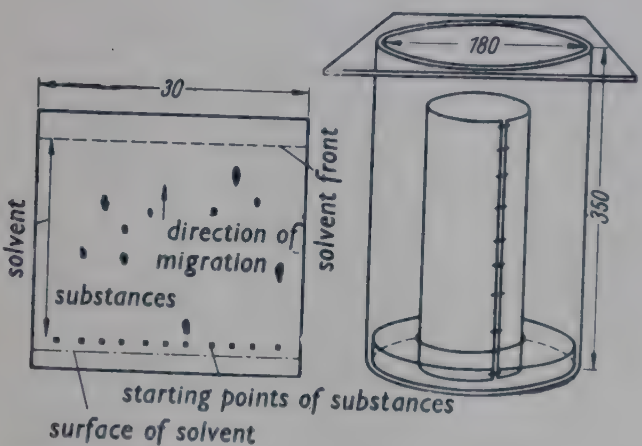


FIG. 20.—Ascending method with cylinder.

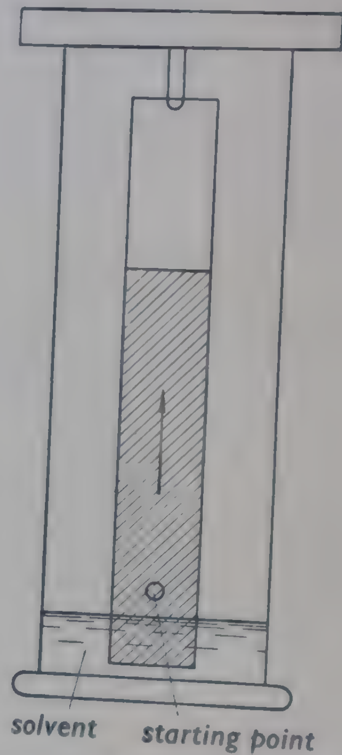


FIG. 21.—Ascending method with suspended strip.

The majority of solvents ascend 20–25 cm. in 2–10 hr. Since the rate of ascension falls off rapidly with increasing height, strips longer than 35 cm. are impracticable for ascending chromatograms.

The ascending method may also be used for preliminary test experiments in test-tubes (Dunn).

10. Chromatography on circular filter-paper

Chromatography may also be carried out on circular filter-paper. In this case the solvent is applied at the centre, from where it soaks out in all directions (229, 268, 201).

Two parallel incisions are made to the centre of a sheet of circular filter-paper so as to produce a narrow radial strip, which may then be hinged downwards from the centre. The substance which it is proposed to run is now applied to the centre of the filter, and the paper is placed on a crystallization dish with the tongue dipping down into the solvent covering the bottom of the dish. The solvent is thereby sucked up and the chromatogram runs so as to produce concentric rings of the individual substances.

In the case of the larger circular filters, the rate of solvent supply by this method is too slow, and the following procedure must be adopted (Zimmerman and Nehring):

A circular filter of diameter the same as that of a desiccator—i.e. about 30 cm.—is laid down on to the flange of the desiccator. The cover, fitted with a one-holed bung, is then jammed down on top of it. The bung is provided with a pipette which has been cut off at the middle and drawn out to a capillary. The capillary should end a few mm. above the surface of the paper and permit of the passage of some 10–12 drops per minute. The solution containing the unknown is, of course, applied to the centre of the filter at the very beginning. The solvent for running the chromatogram is introduced into the reservoir of the pipette, and the head of liquid here then ensures a constant speed of dropping.

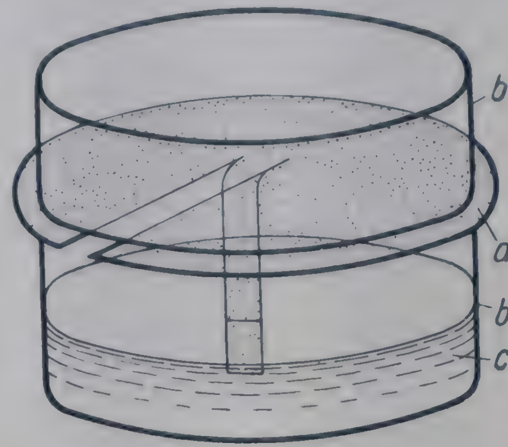


FIG. 22.—Circular filter-paper chromatography. a = filter circle, b = glass dishes (these may be replaced by plates, the lower one being pierced by a hole), c = solvent.

In our laboratories we have resorted to the following artifice to prevent splashing and to counteract irregular dropping speed (F. Weygand, private communication):

After the solution of unknown has been applied and has dried, a small hole is pierced at the centre, into which a tightly rolled piece of filter-paper 1·5 cm. long and about 2 mm. thick is inserted, so that equal lengths are left protruding on both sides. The solvent is then allowed to run down on to this roll, any excess being caught in a vessel placed underneath. In this way the formation of drops of liquid on the surface of the paper is entirely avoided.

FIG. 23.—Circular filter-paper chromatography using the dropping method (362).

The circular paper may also be supported using rings of plexiglass (22).

The substances run out into ellipses as a consequence of the fibre direction in the paper.

H. Pfitzner has informed us of the following technique:

A sheet of filter-paper, 250 × 250 mm. (Schleicher & Schuell 598G), is placed between the plane ground surfaces of two equal-sized plates of glass (7 mm. thick).

The upper plate is pierced by a circular hole of diam. 6 mm. The nozzle of a pipette (capacity about 5 c.c., ext. nozzle diam. 4 mm., int. nozzle

diam. 2 mm.) is inserted into this hole. The pipette is supported by a tripod bent out of wire and fitted with a guide-ring.

After applying the solution of substances through the hole by means of a capillary tube, the pipette is charged with solvent, fitted in the tripod and pressed lightly on to the paper. The speed of effluence is then automatically controlled by the absorbing power of the paper and may, if necessary, be regulated by adjusting the pressure of the nozzle against the paper.

If it is desired to run several substances for comparison simultaneously, the solution to be investigated is, instead of being applied directly at the centre, brought on to some point on a concentric circle near the centre. After running, the compounds in each mixture are then to be found on the respective sectors of the circle (201).

The circular filter method offers the following advantages:

1. The substances run extremely quickly. A chromatogram of diameter 20 cm. has completed running in about 2 hr., one of diameter 32 cm. in 4–5 hr.

2. A surprisingly high separation effect is achieved (*v.* Fig. 24 and

FIG. 24.—Ring chromatogram of amino-acids (362). From inside to outside: histidine, glycine, proline, tyrosine, leucine.

coloured plate IV). Separation is essentially sharper than for the ascending and descending methods. This is no doubt due to the fact that as the substances spread out they become progressively diluted. After what has been stated in the theoretical section, it will be realized that this must result in an increased band sharpness.

3. The interpretation of results is very considerably facilitated, since it is possible to work with several developers by simply cutting out single sectors from the completed chromatogram and spraying each one separately.

4. It is possible to work with relatively large quantities, since each substance runs out into a large ring, thus becoming distributed over a large area.

5. Quantitative evaluation is thereby facilitated, and circular chromatograms may be employed for a quantitative interpretation of results.

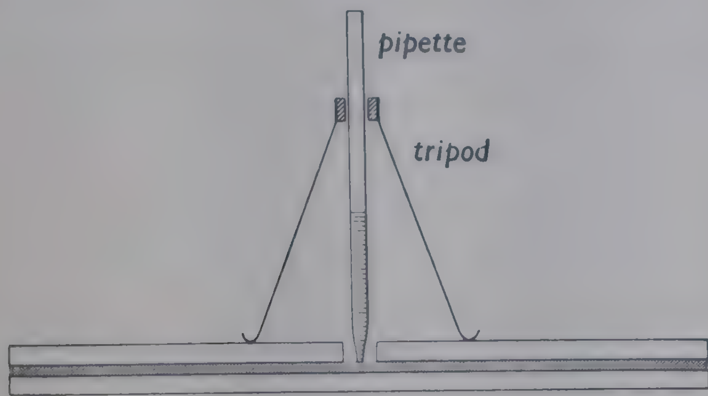


FIG. 25.—Circular filter chromatography (246).

The enumerated advantages of the circular method serve to make this variety a valuable enhancement of chromatographic technique.

11. Drying

When the solvent front has almost reached the end of the paper strip, this is cautiously raised up out of the trough, and the front is marked. At this stage it is important to prevent drops of solvent from the wet upper end from

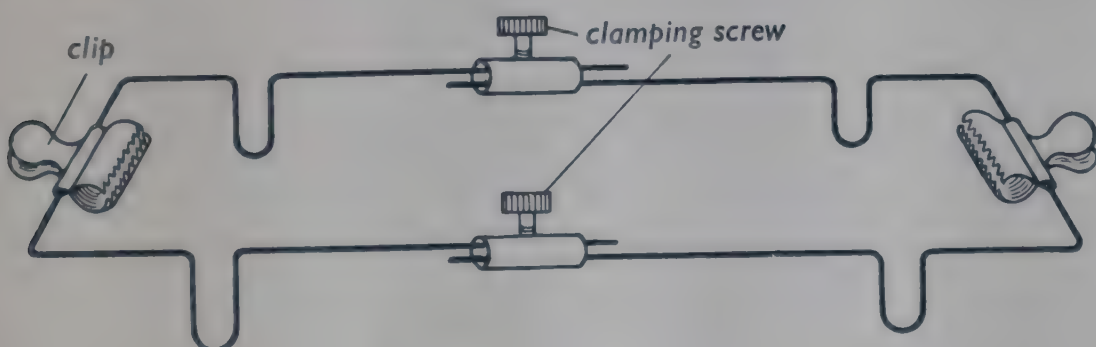


FIG. 26.—Drying frame.

splashing down on to the lower part of the strip. The strip is now hung up on a 'washing line' or clamped in an adjustable drying-frame. According to the nature of the solvent, it is either dried in the air at room temperature or placed horizontally in a drying-oven. It may also be suspended vertically with the starting end downwards, the only important factor being the prevention of surplus solvent from dripping on to the paper. Butanol chromatograms take an hour to dry in the air; phenol chromatograms require the use of a drying-oven (110° C). Drying may also be very conveniently accom-

plished with a hair-dryer, which is the best method of preventing damage to the paper.

12. Developing *

If the spots on the chromatogram are not recognizable as a result of their own colour or fluorescence, it is necessary to develop them by treatment with a suitable reagent. The reagent is sprayed on with an atomizer. A suitable type of atomizer, which may readily be constructed by any glass-blower, is shown in Fig. 27.

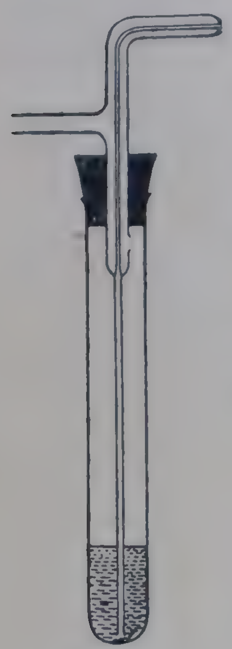


FIG. 27.—Atomizer.

The chromatogram should not be sprayed so liberally that it begins to drip, with a resulting smudging of the spots. After drying, a period in the drying-oven, once more on the frame, is generally necessary for proper development. The use of an infra-red lamp is also advantageous in many cases. A few substances may be rendered visible by exposing to gases or vapours. The individual developers are dealt with in the special section.

13. Evaluation of results

Pencil circles are drawn round the spots on the completed chromatogram, the centre of each spot is marked and the distances centre point of spot–starting point are measured off. Division of these lengths by the distance starting line–solvent front then gives the R_f-values (cf. p. 4).

A key is appended for the qualitative evaluation of results (Key A). This should be employed in the following manner:

The key sheet is placed on the chromatogram so that its start coincides with the line 'start' and the solvent front with the line 'front', the point chosen on the solvent front being on the extension of the line start–substance. The characteristic R_f-value line then crosses the spot produced by the substance in question.

'Keys' of this type may be readily self-constructed for use with any particular substances and solvents, depending on the field of work.

Direct reference to the front is not essential here provided that some known substance has been run simultaneously. The spot from this substance is merely brought to coincide with its appropriate R_f-line, after which the unknowns are to be found each on their respective lines, the corresponding R_f-values then being read off.

An arrangement serving a similar purpose and known as a 'Partogrid' is to be found in the literature (267a).

* The expression 'developing' is employed here in the same sense as in photography—i.e. rendering visible by treatment with appropriate reagents. In adsorption chromatography, however, by 'developing' is understood in contradistinction to this, the separation of a band into a number of different zones.

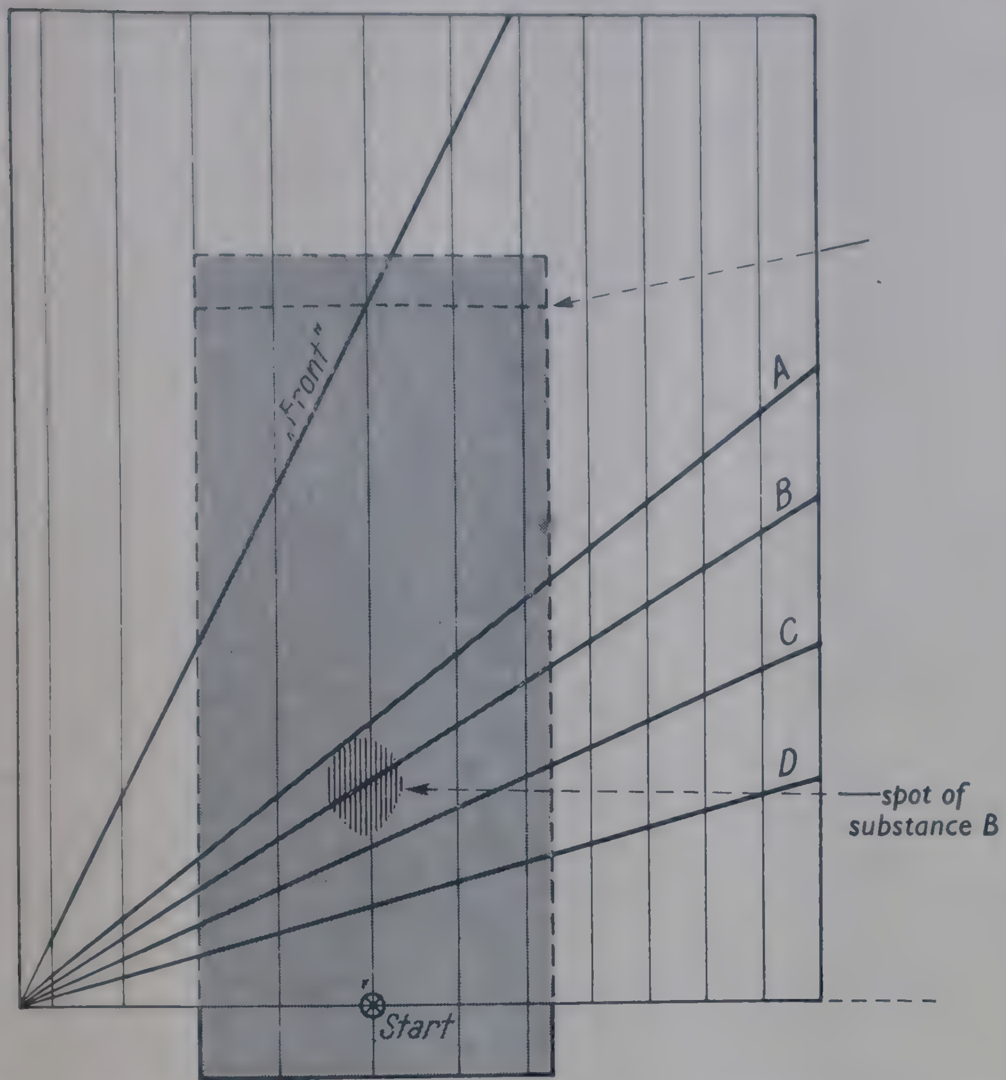


FIG. 28.—Evaluating the results on a paper chromatogram with the aid of a key. $A, B, C, D = R_f$-value lines.

14. The two-dimensional method (29, 68, 91)

If a single solvent mixture fails to give satisfactory results—i.e. a complete separation of every component—resort is made to the two-dimensional technique. The mixture of substances is applied at one corner of a large sheet of paper—30 × 40 cm. or larger—and the mixture is run in the one direction. This produces a perfectly normal one-dimensional chromatogram along the edge of the sheet. The chromatogram is now dried, rotated through 90°, and then run at right angles with a second solvent. In this way maps are obtained which permit of the simultaneous identification of, for example, up to twenty amino-acids. The apparatus used in two-dimensional paper chromatography is shown in Fig. 29. The cabinet may be made of glass, plastic or wood with glass windows. A wooden cabinet must be given a coat of paraffin wax on the inside.

The ascending method is again much easier here. The sheet (30 × 30 cm. = $\frac{1}{4}$ roll) is rolled round to form a cylinder, which is placed in a 4 lit. beaker containing the solvent at the bottom. On completing the run with

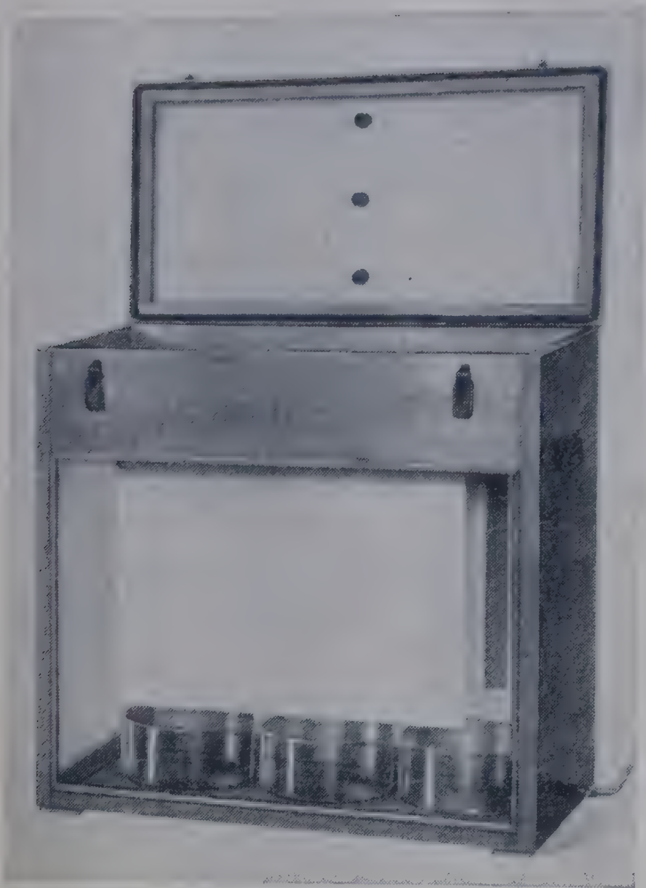


FIG. 29.—Apparatus for two-dimensional paper chromatography. (From the catalogue of Hormuth & Vetter, Heidelberg.)

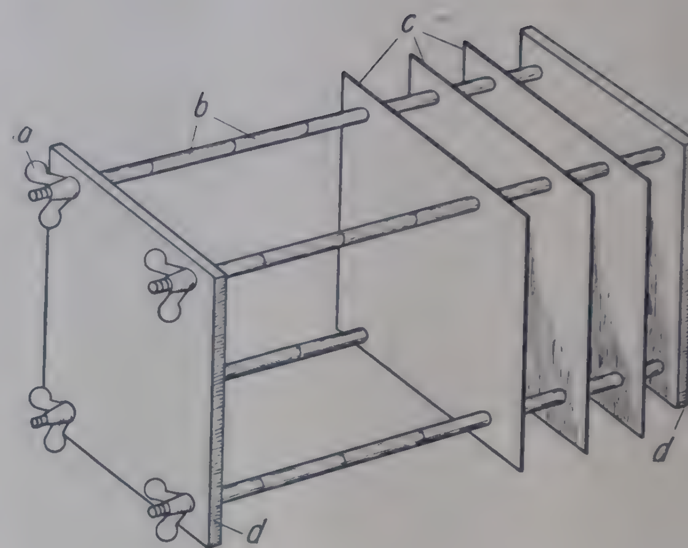


FIG. 30.—Frame for running several two-dimensional chromatograms (91).

a = wing nuts
b = detachable tubes
c = paper
d = cover plates 30 × 30 cm.

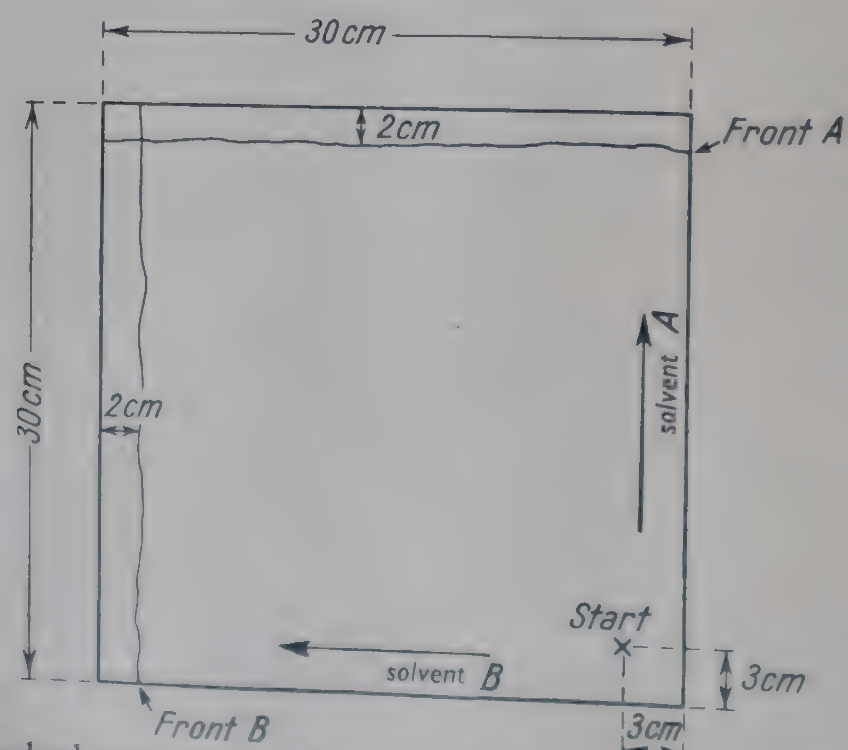


FIG. 31.—Standard measurements for an ascending two-dimensional chromatogram.

the first solvent the paper is dried, rolled round in the other direction and run once more.

Several ascending two-dimensional chromatograms clamped in a frame may be run simultaneously (91).

When working with the two-dimensional method, which represents the standard method for amino-acids, adherence to the measurements given in Fig. 31 is to be recommended. It is for these measurements that the appended key B is intended.

Not until a $\frac{1}{4}$ roll has been found to give an insufficiently sharp separation should resort be made to the descending method.

Quantitative Evaluation

Although paper chromatographic work is carried out using extremely minute quantities of substance, micro-analytical techniques have been developed permitting of a quantitative estimation of the substances on the chromatogram. It is, indeed, not surprising to learn that this process, conditioned as it is by the small quantities available, is usually associated with errors of some 5–10%. Nevertheless it often provides valuable information concerning the quantitative composition of natural products.

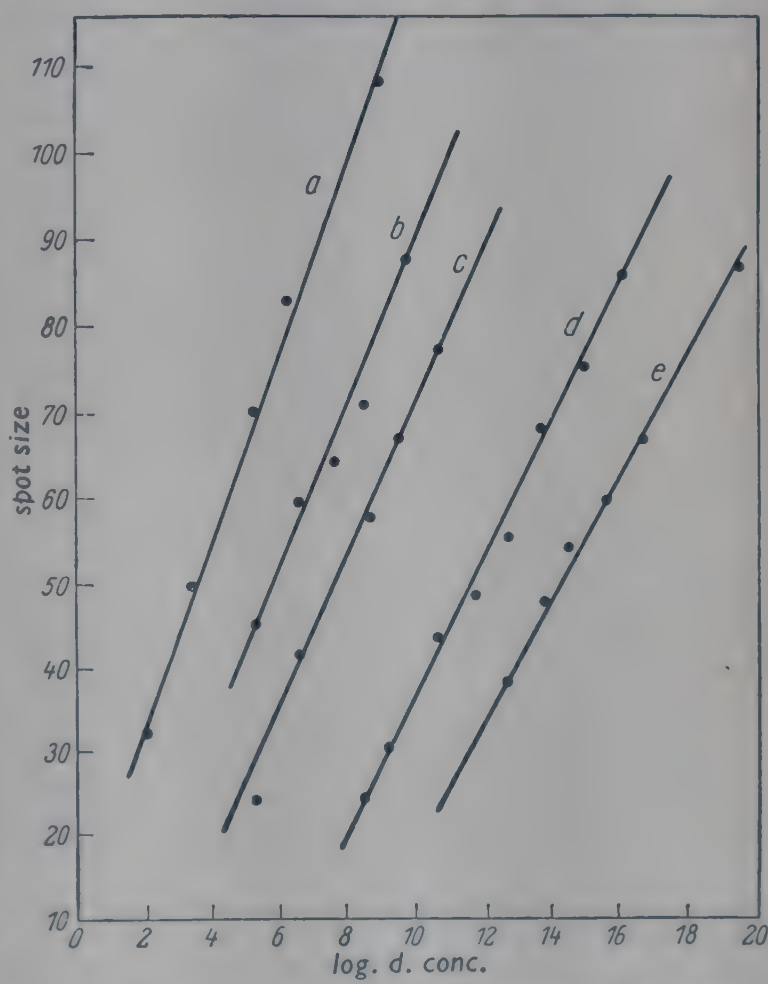


FIG. 32.—Linear relationship between the size of the spot on the chromatogram and log. d. concentration (115).

1. Size of spot

The area of a spot produced by a substance is proportional to the logarithm of the quantity of that substance. Thus if equal volumes of a standard solution and an unknown solution are chromatographed together, the quantitative composition of the unknown solution may be deduced to an

accuracy of 10% (115). The area may either be determined with a planimeter or by cutting out the paper covered by the spot and then weighing it (16).

The spot dilution technique may also be employed. In this a number of solutions of progressively increasing dilution are run simultaneously with the unknown solution. A scale of spots is thus obtained, and it is not a difficult matter to fit in the unknown spot at some point in this scale. The concentration in the unknown may then be given with an accuracy of about 50%, which, if nothing else, often provides quite valuable indications.

2. Photometry

The chromatogram is developed with a suitable reagent, and the intensity along the strip is subsequently measured with a photometer, and a graph plotted (52, 116, 121, 228).

In principle any photometric colorimeter is suitable for this purpose. A suitable arrangement for evaluating electrophotograms is given by Grassmann (134). A more detailed account of this is to be found in the section on paper electrophoresis. The photometric method entails a considerable capital outlay, but is by far the most convenient procedure, allowing of a fair degree of accuracy, and for this reason it is preferred for serial investigations. The chromatogram is simply drawn past the window of a photocell, the readings being plotted on a graph.

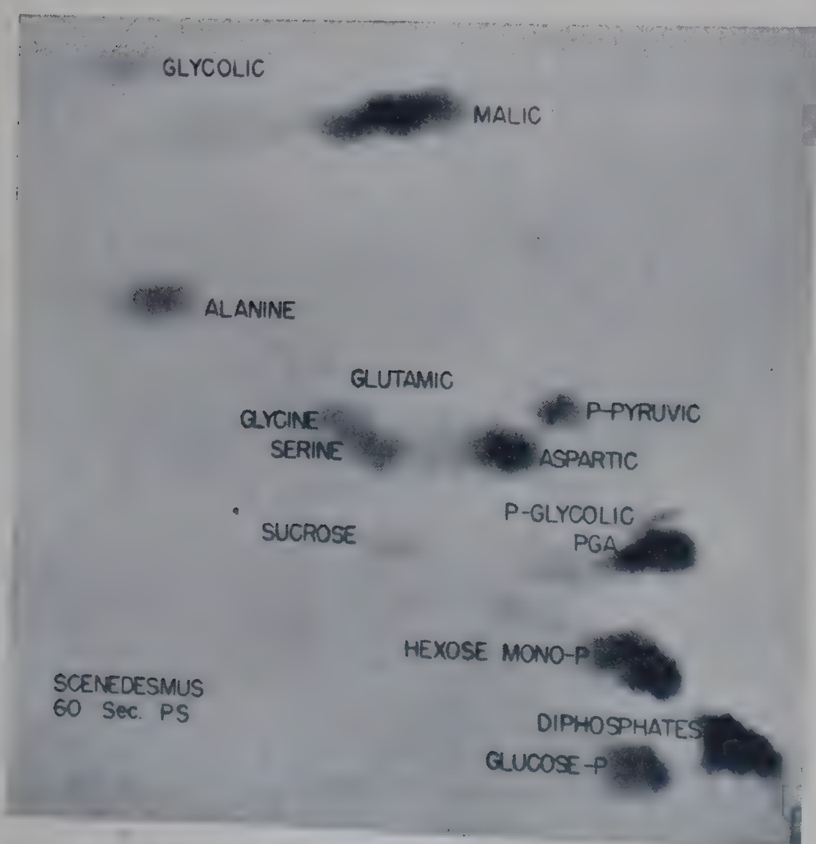


FIG. 33.—Autoradiogram of the products of CO_2-assimilation with $^{14}CO_2$.

Kindly placed at our disposal by Dr. A. A. Benson.

3. Autoradiography

This is only possible for radio-active materials; all that is necessary is to lay the dried chromatogram on to photographic printing paper or to go over the strip with a Geiger-counter (61, 62, 112, 175, 176, 197, 300, 304).

A noteworthy success in researches on carbon assimilation was achieved by Benson and Calvin (18) with the aid of ^{14}C and ^{32}P.

The following radiographic technique was employed:

For exposure the dried radiochromatogram is folded, with the starting point of the separated mixture in one corner, so that it just covers a normal Eastman Kodak X-ray film. One or two films, as described below, are exposed to the radiochromatogram in a light-tight Eastman Kodak X-ray film exposure frame. The film should be pressed evenly against the chromatogram with a carton filled with sand. If the chromatogram contains both ^{14}C and ^{32}P, then a film coated with emulsion on both sides is employed or two ordinary films packed in one after the other, with the emulsion side facing the source of rays, and pressed on to the chromatogram. When packed in this way, the ^{14}C produces a latent image only in the directly adjacent emulsion, whereas the ^{32}P gives rise to an image capable of being developed in both emulsion coatings. The latent image in the more distant emulsion is thus to be attributed to the action of ^{32}P.

In these experiments the radioactivity proceeding from Whatman No. 1 filter-paper amounted to 30% of that which is registered if the radioactivity of the sample, spread out and dried in a thin film on a glass or metal base, is measured directly. The period of exposure of an X-ray film is a function of the radiation proceeding from the paper. According to Calvin, 15,000 disintegrations per minute per cm.^{2} give a satisfactory latent image in three to six days.

4. Extraction of the spots

When followed by a micro-estimation, this method should lead to the most accurate results (117, 230). First of all, however, it is necessary to locate the substances on the paper. Development cannot be considered, since the substance is destroyed or transformed into some derivative in the process. It is therefore necessary to proceed as follows:—

(*a*) Commence developing cautiously with a much-diluted reagent (230). This leads, however, to loss of material.

(*b*) Several chromatograms are run simultaneously on a broad strip of paper, one being afterwards cut off and developed, from which the positions of the spots on the others may be inferred (Fig. 34).

(*c*) A strip chromatogram is, however, more suitable. For this a whole strip of solution is applied to the start line instead of the usual single spot. In this way it is possible to work with a few mg. of substance, a quantity which may be determined by means of normal micro-technique.

An even application of the strip may be achieved after some little practice by running a fine pipette directly over the

FIG. 34.—Dissection of paper chromatogram for subsequent quantitative determination.

Only one strip is developed. The others are then cut out and extracted. The individual components are determined in the solutions thus obtained.

paper. The paper may also be attached to the drum of some registerin instrument which then gradually rotates while a slowly dischargin pipette is applied to its surface (352, 227).

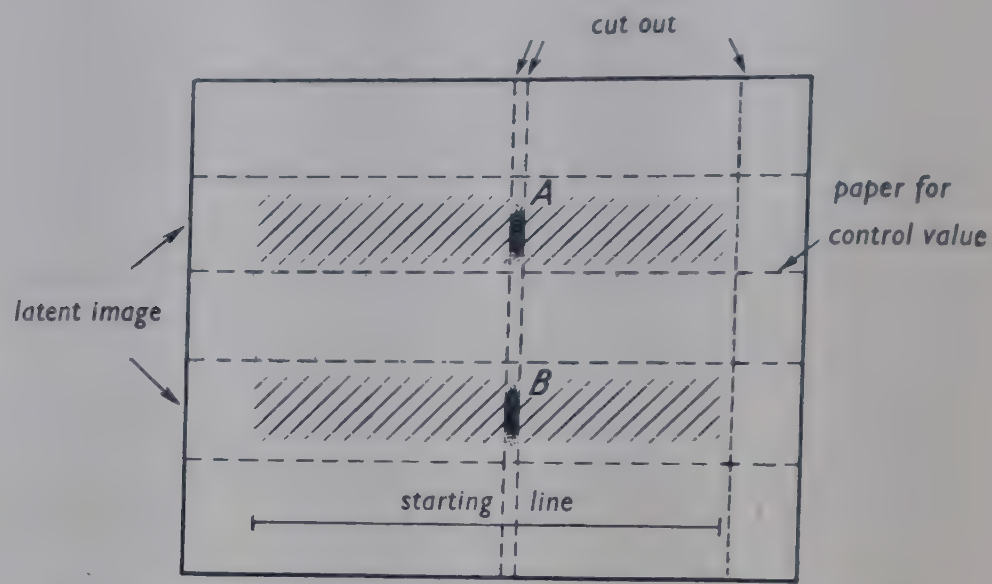


FIG. 35.—Strip chromatogram for quantitative evaluation.

A narrow strip is cut out from the middle and developed. The substances are thus located, cut out and extracted in a suitable manner.

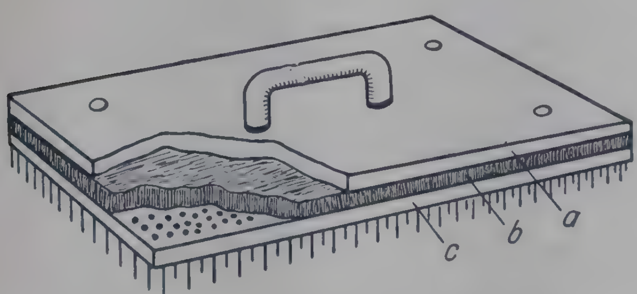


FIG. 36.—Wire brush for applying developer.

a = backing, b = rubber, c = plastic. Distance apart of needles 7 mm. Length of projecting needle 10 mm., diam. 0·7 mm. The needles should have a certain amount of play in the holes (30).

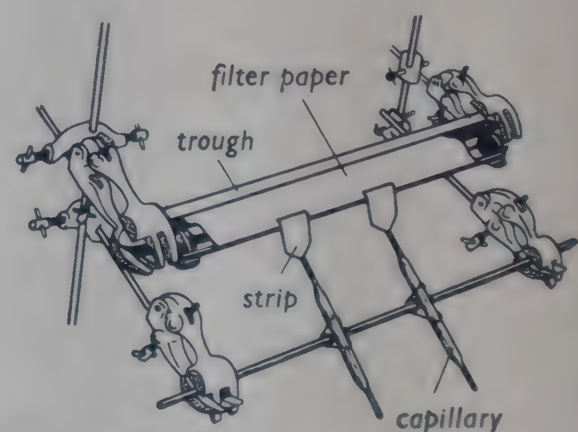


FIG. 37.—Arrangement for transference of substances from the chromatogram strips into capillaries (70).

It is, however, easier to employ the artifice (234) of folding the paper along the starting line and then dipping into the solution being investigated.

(*d*) The paper is pricked with a wire brush charged with the reagent (30). The brush, which should have only 3–4 bristles per cm.², is dipped into the reagent so as to wet only the tips of the bristles, and then pressed down on to the paper. In this manner a coarsely pricked pattern is obtained on developing, on which the spots may easily be recognized, but in spite of this no appreciable loss of material ensues.

This process is indispensable for two-dimensional chromatograms, since it is not possible to run substances for comparison in these.

After locating the positions of the spots in the manner described, the respective portions are cut out and the substance to be estimated is washed out (Figs. 37 and 38; cf. also 349).

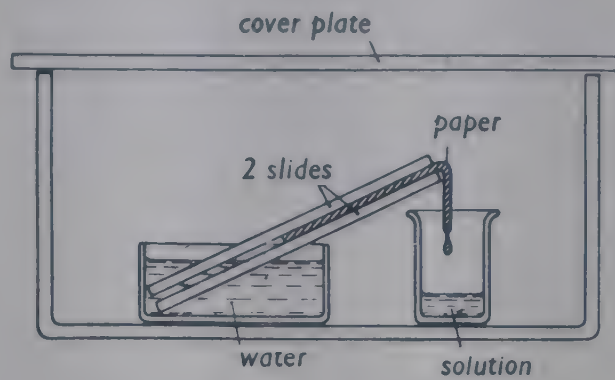


FIG. 38.—Extraction of paper cut out from the chromatogram for subsequent quantitative micro-determination.

The paper is placed between two microscopic slides, the substance being extracted by the water thus sucked up (88).

A combination of both methods is given by Sanger (273).

The paper strip is cut to a point and placed between a pair of microscopic slides. The strip ends with its point at the opening of a capillary tube which sucks in the solution of substance washed out of the paper.

The solutions of substances obtained in this manner are then estimated by suitable micro-methods.

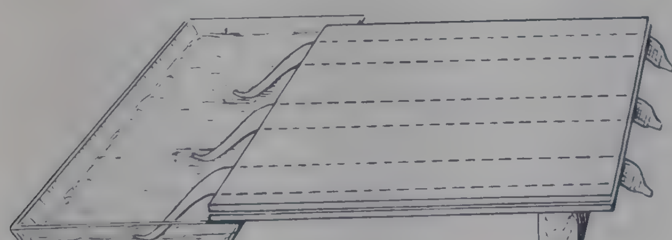


FIG. 39.—Simultaneous extraction and concentration may be achieved in the following manner (87): The strips from a strip chromatogram are cut to blunt points at one end. The strips are then placed between two glass plates with both ends projecting, the broad ends dipping down into a bath of distilled water, the pointed ones hanging freely in the air. Each strip then draws up water until it is saturated and the water evaporates away at the projecting points. A continuous flow is thus established towards these points as a result of which the substance is washed along into the tips in the course of a few hours.

5. **Retention analysis** (327, 330, 333, 335)

If a solution be permitted to suck in from one end of a paper strip, the dissolved molecules advance along a front perpendicular to the direction of suck. If a part of this front encounters some barrier, for example a substance which reacts with the dissolved molecules, this part remains behind the section which is advancing unimpeded, thus giving rise to a gap. At the point of obstruction, the impeded portion will not start advancing again until

the molecules causing the disturbance have all reacted. The area of the ga in the front thus represents a measure of the number of molecules retainin the advancing molecules (Th. Wieland).

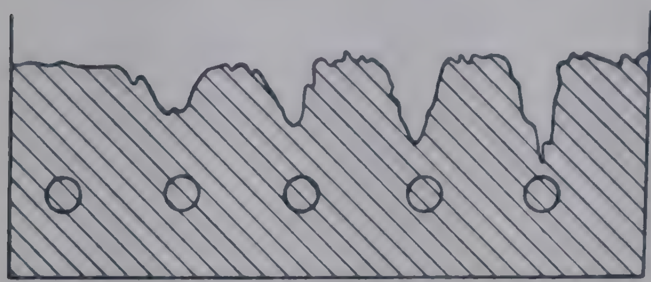


FIG. 40.—A retentiogram of various quantities of glycine applied directly to the filter paper (327).

Left to right 0–2·5γ. The method is evidently of extreme sensitivity.

This process lends itself partic ularly well to an estimation o amino-acids on paper chromato grams. Amino-acids combine with Cu-ions to form a copper complex and thus retain Cu-ions in ascending copper salt solution. Hydroxy carboxylic acids, purines, and in principle a great many other substances, may be estimated quantitatively on the paper chromatogram in this manner.

The dried strip of a one-dimensional amino-acid chromatogram is rolled round into a broad cylinder and stood 3 cm. deep in a 0·1% solution of copper acetate in 10% aqueous tetrahydrofuran to which a little glacial acetic acid has been added to avoid

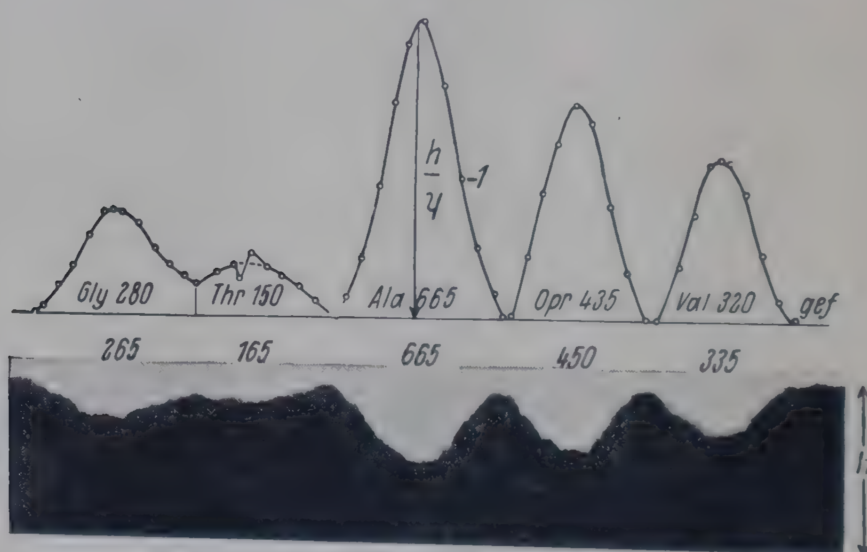


FIG. 41.—Evaluation of a paper chromatogram of threonine, alanine, hydroxyproline (Opr) and valine (applied in strips), with phenol as organic phase.

The areas under the curve shown in the same diagram, obtained by reduction of the ordinates of the points of maximum retention to O, are in the ratio 280 : 150 : 665 : 435 : 320. The ratio of the amounts actually weighed in was 265 : 165 : 665 : 450 : 225.

turbulence resulting from basic acetate (187). The Cu-solution then advances up from the broad side and is retained at points corresponding to the distribution of the amino-acid. When the Cu-solution has risen about 5 cm. in the solvent-saturated atmosphere and has almost reached the upper edge of the paper, the strip is dried for a short period in the air and then

sprayed with a 0·1% solution of thiocyanic acid in 10% acetone. As a result of this treatment, the copper-containing portions of the surface become clearly visible.

It is also possible to employ a radioactive Cu-salt solution, the resulting retentiogram then being investigated radiographically (330).

FIG. 42.—Radioautogram of an amino-acid retentiogram (330) of equimolecular quantities of glutamic acid, alanine and arginine.

Preparative Paper Chromatography, Chromatography on Cellulose Columns

Under suitable experimental conditions paper chromatography also lends itself to work on a scale of 0·5–1 g. per substance. On ordinary paper strips, however, it is only possible to work with a 100th part of the quantities generally employed for preparative purposes.

1. Strip chromatogram. See p. 33.

2. Use of the 0·6-cm.-thick American paper 'Schleicher & Schuell 470A', which is about twenty times as thick as 'Whatman' paper, enables twenty times the quantities of substance to be chromatographed (227). The thick paper cannot, however, be suspended directly in the solvent because this would be sucked up into the entire paper in the course of a few minutes. A 9-cm. strip of 'Whatman No. 1' is therefore sewed on to the upper end of the thick paper, thus ensuring a supply of liquid at the correct rate.

'Schleicher & Schuell' also supply a thick card consisting of the same material as paper 2043b.

3. Preparative paper chromatography using columns consisting of 200–500 circular filter-papers on top of each other ('chromatopile') has proved most useful. The solution is first absorbed on to ten circular filters, which are allowed to dry, covered by twenty 'empty' filters, and then placed on top of a whole column of filter-paper (218).

In order to discover the position of the various zones when working up, a line of reagent may be brushed down the side of the column, using a hair

pencil—as in adsorption analysis—the 'full' filter-discs located in this mann then being extracted. Alternatively, the zones may be washed through, tl separate fractions being collected by a fractionating device (e.g. 114) ar afterwards worked up. In either case it is advisable to run an ordinar qualitative chromatogram simultaneously in order to gain a clear idea of th order and number of the anticipated zone The composition of the individually collecte fractions should also be tested on a pape chromatogram.

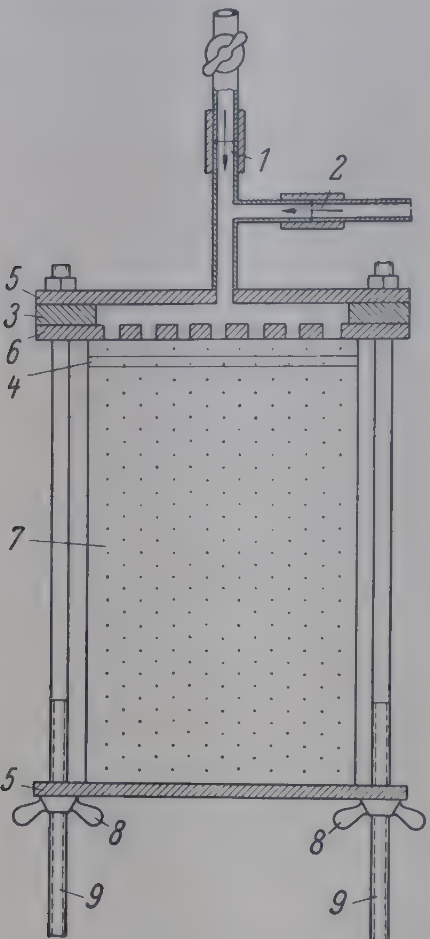


FIG. 43.—Chromatopile.

1. Inlet-tube for filling. 2. Tube for subsequent addition of solvent. 3. Rubber washer. 4. Filter charged with substance. 5. Cover plates. 6. Perforated plate. 7. Filter discs. 8. Wing nuts. 9. Screw thread (17).

4. In an analogous manner, a sheaf o fifty paper strips of the normal dimensions– i.e. about 12 × 50 cm.—may be packe together between two glass plates and thu used as an ascending chromatogram ('Chroma topack') (255). This method offers the ad vantage of allowing the subsequent removal o one of the strips which, when developed gives a direct picture of the position of th zones.

5. The best method here is undoubtedl that offered by chromatography on cellulos powder, which, as it happens, is also cheape than the other methods described (159). In principle the technique employed is the sam as for ordinary adsorption chromatography.

The following cellulose powders ar especially suitable:

1. 'Solca floc', manufactured by Brown Berlin (U.S.A.).

2. 'Zellstoffpulver' for chromatographic purposes, manufactured by Zellstoff-fabrik Mannheim-Waldorf (Germany).

3. Whatman and Schleicher & Schuell also supply paper powder (No. 123).

The powder may be filled dry into a glass chromatograph tube, fitted with a sieve plate at the base and afterwards covered by an ordinary filter at the top, and then treated with the solvent so as to form a solid sludge. It is, however, better to stir into a sludge at the start, afterwards pouring as evenly as possible into the column, sucking gently all the time so that the mixture settles uniformly. A uniform column filling is here, as on other occasions in chromatographic work, a pre-condition for clean work.

Chromatography on columns of cellulose powder has the advantage of giving the same R_f-values as are obtained using ordinary paper chromato-

grams. Thus whenever a separation on a micro-scale on paper has been achieved, it is possible to proceed straight on to the column.

The best method of working up the substances on the column is to continue with the addition of solvent until the substances sought flow out at the

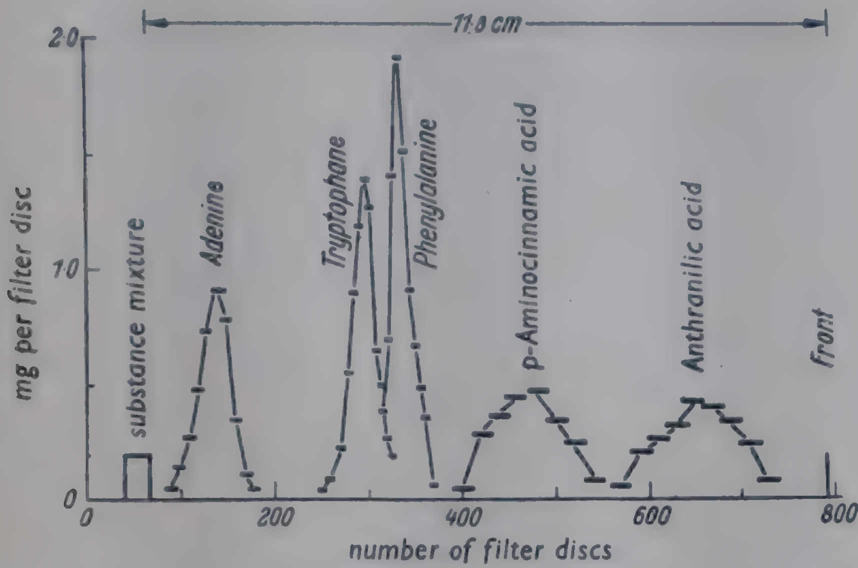


FIG. 44.—Graph of distribution in a chromatopile.

bottom, where they may be collected separately with the aid of a fraction collector. The composition of the individual zones discharged may, as usual, be ascertained on paper chromatograms.

6. It is not proposed here to deal with distribution chromatography on starch. For an account of this the original literature should be consulted (289, 290, 297).

Paper Electrophoresis

It has been demonstrated that the electrophoresis of charged molecules may readily be carried out on filter-paper (Th. Wieland, F. Turba). This elegant method, which has already proved of very considerable value not only in chemistry but also in medicine, in the investigation of protein mixtures, naturally greatly facilitates all work involving electrophoresis. This may

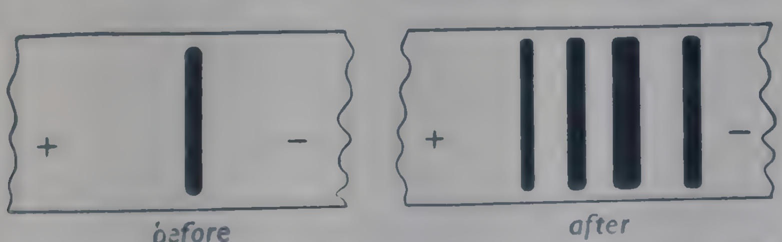


FIG. 45.—Paper electrophoresis of a substance mixture applied in strip form.

now be carried out with an apparatus like that of Tiselius, which, in spite o its remarkable simplicity, still gives results of great exactitude (80, 100, 14 306, 327, 328, 331, 333, 334).

A strip of filter-paper is saturated with a suitable buffer solution whic has been adjusted to the isoelectric points of the components of the particula mixture being investigated. This strip of paper is suspended in a dam chamber with its two ends dipping into buffer solutions which are connecte up to the anode and cathode respectively. On applying a suitable tension— as a rule 100 V—electrophoresis commences. After running for 5–10 hr the current is interrupted and the pape is removed, dried and developed in th usual manner.

Proteins may be identified by thei property of strongly absorbing certain dyestuffs—e.g. azocarmine B, bromophenol blue, amido-black 10 B and sublimate. It is preferable, especially if it is intended to follow up with a quantitative estimation, to apply the solution in the form of a streak, as opposed to a circular drop (333).

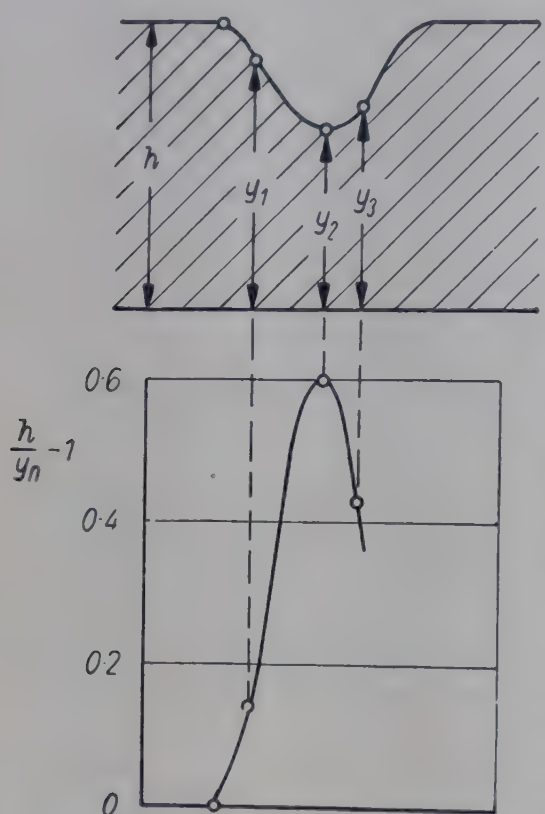


FIG. 46.—Graphing the retention gaps (333).

The protein solution is either applied with a pipette or a hair-brush which is drawn across an oblique pencil line on the dry paper. After drying for a short time, the strip is sprayed uniformly with the same buffer solution as in the chamber. The paper may also be treated with the buffer first, the protein solution then being applied after having pressed out excess buffer.

A quantitative estimation of the substance may be made by a colour comparison or by subsequent elution followed by colorimetric analysis.

A method of evaluation is also provided by retention analysis (333).

Since in the paper electrophoresis of proteins the ascending Cu-ions are retained by columns of substances as opposed to spots, a change in the method of calculation is necessary (cf. p. 36). This is demonstrated once more in Fig. 46.

Before the protein electropherogram is submitted to retention analysis, the buffer solution must be washed out of the paper after having first fixed the protein with azocarmine B (306). This acid-wool dyestuff precipitates the protein as a water-insoluble salt, which is nevertheless still capable of retaining the copper. Using this process, it is possible to achieve a separation of the constituents of a most varied series of specific peptides and proteins— e.g. those of the *Amanita vulgaris* (331) and of the cobra poison (333).

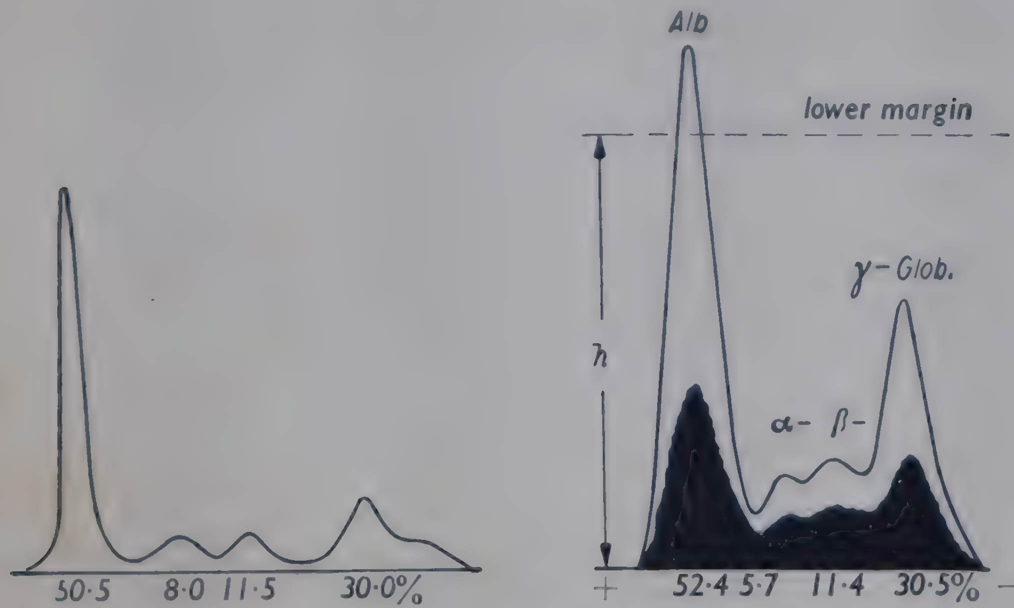


FIG. 47 (333).—Plasmocytom serum.

Compact curve on right: shadow outline of original retentiogram, above: transformed curve. Left: the same serum analysed in the Tiselius apparatus.

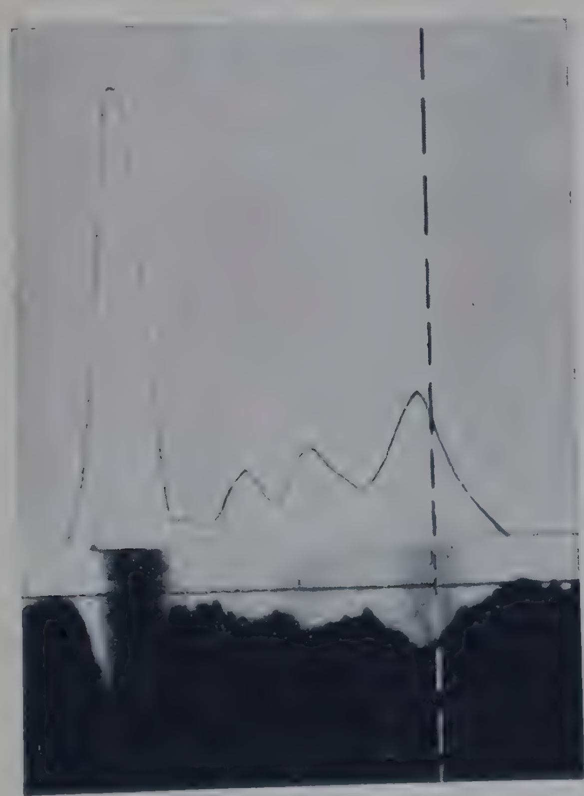


FIG. 48 (333).—Pherogram of normal serum.

Below: view of original; above: transformed concentration curve. The vertical dotted line shows, as in the other diagrams, the position of the starting line of the protein mixture.

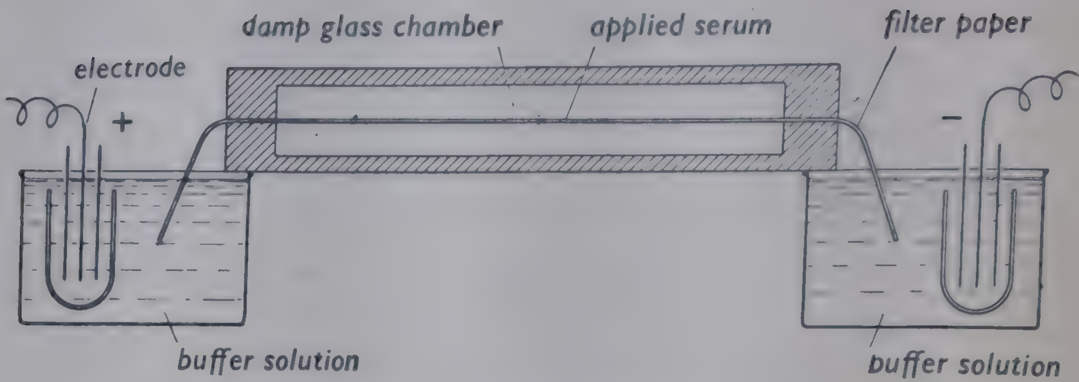


FIG. 49.—Scheme of chamber (134).

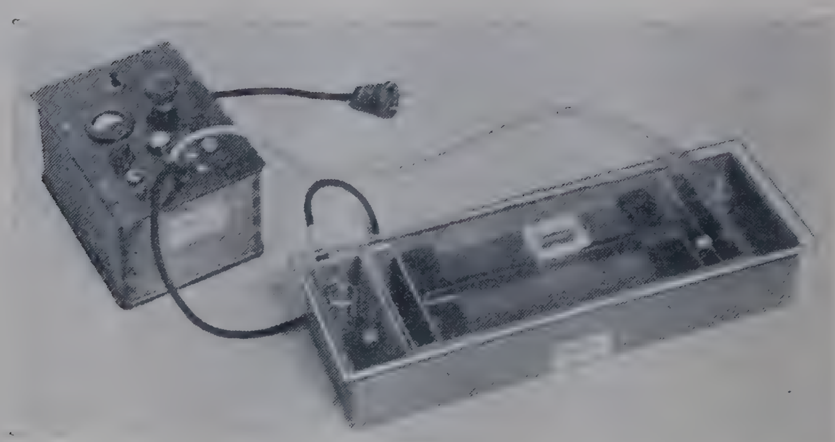


FIG. 50.—Grassmann's electrophoresis chamber. (Taken from *Med. Monatsschrift*, 10, 707 (1951).)

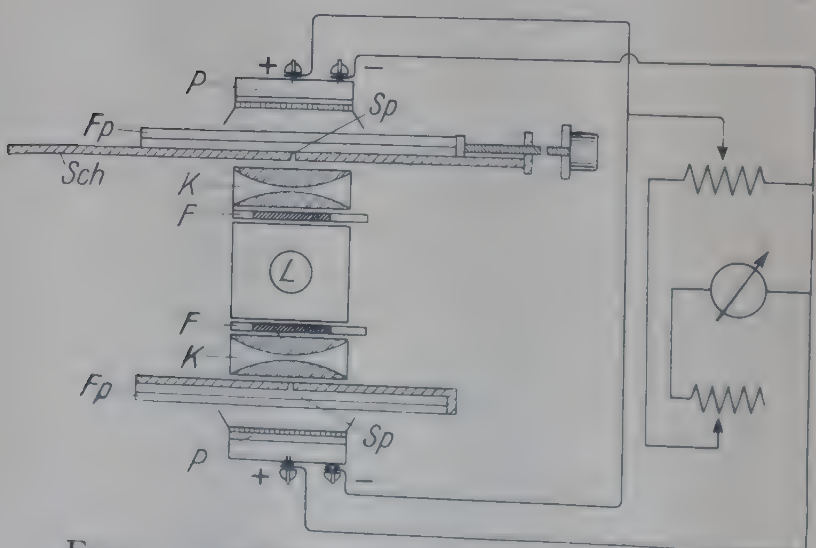


FIG. 51.—Scheme of photometric evaluation apparatus (134).

L = light source, *F* = filter, *K* = condenser, *Sch* = stage, *Sp* = slit, *Fp* = filter paper, *P* = photo-cell.

For clinical practice, Grassmann *et al.* have developed a special technique which is both reliable and versatile (134, 135):

In the course of an extensive series of comparative experiments with a large number of dyestuffs, it was shown that amido-black 10 B (manufactured by 'Farbenfabriken Bayer' serological division) was superior to all dyestuffs hitherto employed. Dyeing is much more intensive than with azocarmine or bromophenol blue, which thus

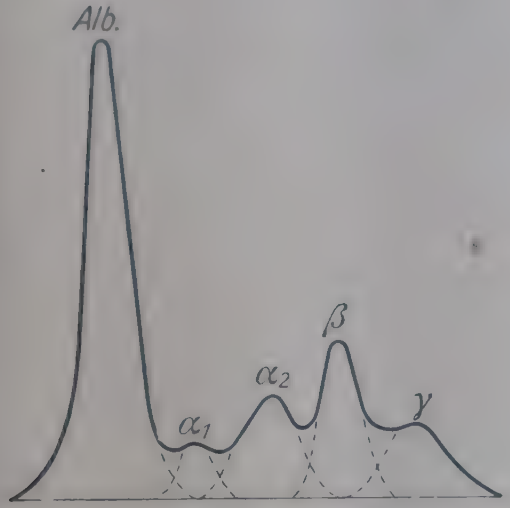


FIG. 52*a*.

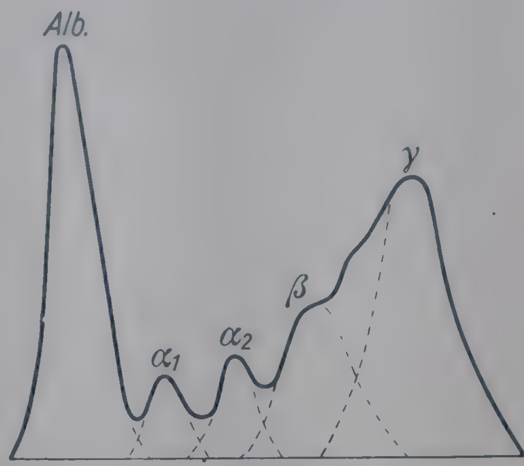


FIG. 52*b*.

permits of work with even smaller amounts of serum. The fractions obtained in this way are also sharper.

On account of the high protein affinity of amido-black 10 B, the intensity of dyeing, within the concentration range considered, is almost exactly proportional to the quantity of protein.

Direct photometric evaluation on the strips leads to useful results only if these are rendered largely transparent by immersion in a solvent having the same refractive index as the fibres in the paper. An outline description of the apparatus and short instructions for its use follow:

0·01 c.c. gastric juice (which corresponds to 0·4–1 mg. protein) is applied very evenly in a 3-cm.-long oblique streak to a strip of Whatman No. 1 filter-paper (4 × 30 cm.) which has been saturated according to the method of Michaelis with a veronal–sodium acetate buffer of p_H 8·6 and ionic strength U = 0·1. This paper is freely suspended horizontally in a damp glass chamber with both ends dipping into vessels containing the same buffer solution. The buffer solution is connected with electrodes of platinum or carbon via a labyrinth (to avoid p_H displacements) (cf. Fig. 49).

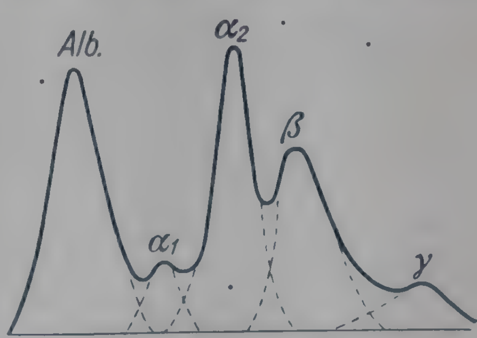


FIG. 52*c*.

Electrophoresis is carried out at room temperature in order to suppress errors due to evaporation. About 2 mA flows on applying a direct current of 110 volts, and the individual protein fractions in the serum migrate to the anode at speeds governed by their velocity of migration. In 12–15 hr. an adequate separation will have been achieved. The strips are then removed, dried at 100° C. in a drying-oven and dyed by agitating for 10 min. in a bath containing a saturated solution of analytically pure amido-black 10 B in methanol containing 10% glacial acetic acid. To remove excess dyestuff, the strip is repeatedly washed with a mixture of methanol and 10% glacial acetic acid until the protein-free portions of the filter-paper possess only a light blue tinge. After drying in the air, the strip is saturated with a mixture of paraffin oil and α-bromonaphthalene (of refractive index n_D = 1·51), as a result of which it is rendered

practically transparent. It is then laid between two plane-glass plates, taking care to exclude all bubbles, and drawn past a slit about 1 mm. wide and 40 cm. long, being illuminated from the front by a parallel beam of light. After traversing the strip, the light-beam passes into a photo-electric cell; by using a compensating circuit, of the type usually employed with the Lange colorimeter, it is possible to arrange for the deflections on the instrument to give the extinction value directly (cf. Fig. 51).

A protein-free but otherwise similarly treated strip, serves as a 'blank' in the compensation circuit. With this contrivance the evaluation of results on a strip requires no longer than 5 min. The apparatus may also be coupled with an automatic recording device. The extinction values measured are then plotted as a function of the distance along the strip, and curves similar to those resulting from the Tiselius method are obtained. The areas are then measured off on the completed Gauss curves; these areas give the relative percentages of the individual fractions. By summation followed by calibration using solutions of known protein content, the total protein content may thus be determined.

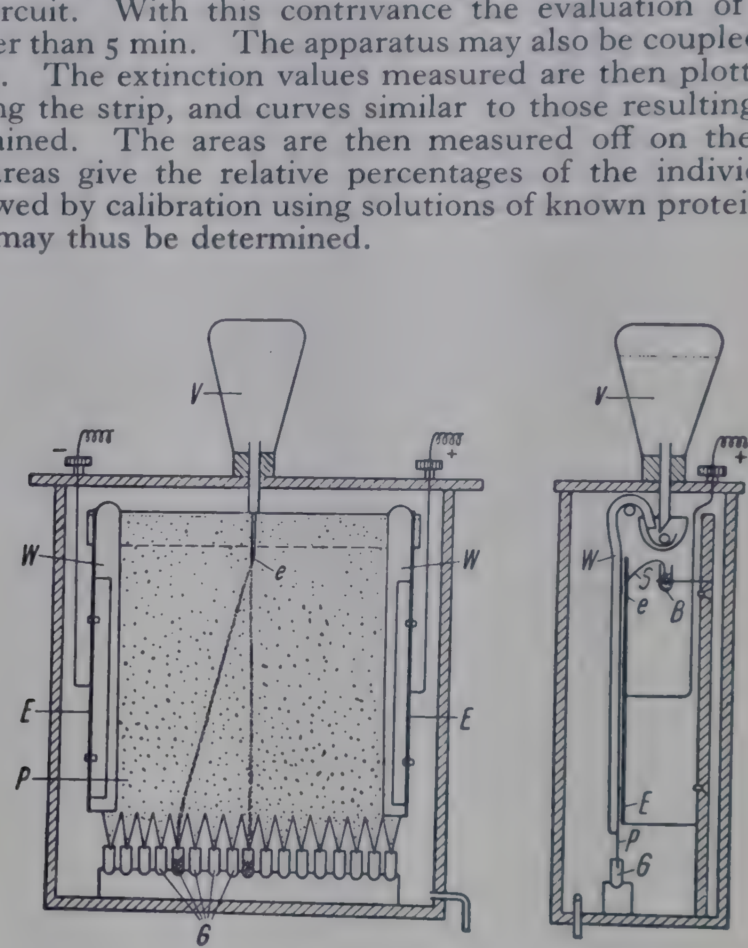


FIG. 53.—P = paper, E = electrodes, W = wick of cotton wool, V = reservoir for buffer solution, e = inlet for solution of substances, B = solution of substances, S = paper tongue, G = collecting vessel (132).

Fig. 52 shows curves obtained by means of this method for three typical cases—viz. serum from (*a*) a clinically healthy subject, (*b*) a subject suffering from nephritis, (*c*) a subject suffering from cirrhosis of the liver. The results of a planimetric evaluation of the curves for the five fractions (albumin and the four globulins α_1, α_2, β, γ) separated are appended:

Alb.	60·1%	Alb.	29·3%	Alb.	33·7%
α_1	4·4%	α_1	4·1%	α_1	5·7%
α_2	11·9%	α_2	6·9%	α_2	27·2%
β	13·3%	β	14·7%	β	21·4%
γ	10·3%	γ	45·0%	γ	12·0%

Paper electrophoresis by means of a continuous flow method (132, 133) enables larger amounts of material to be separated in the simplest manner imaginable. The mixture to be separated is allowed to flow into a buffer solution, which is slowly streaming down the paper at right angles to the lines

of force of an electric field. Charged particles thereby flow in a direction which is a resultant of the velocity of flow of the buffer solution and the velocity resulting from the application of the electric field at right angles to this. Particles of differing electrophoretic mobility are thus discharged at different points at the lower end of the sheet of paper.

The apparatus is shown in Fig. 53. The chamber has the dimensions 22 : 26 : 7. The sheet of paper (Machery & Nagel No. 819, Schleicher & Schuell, 2040a or 2043a) with the buffer solution running down it is clamped

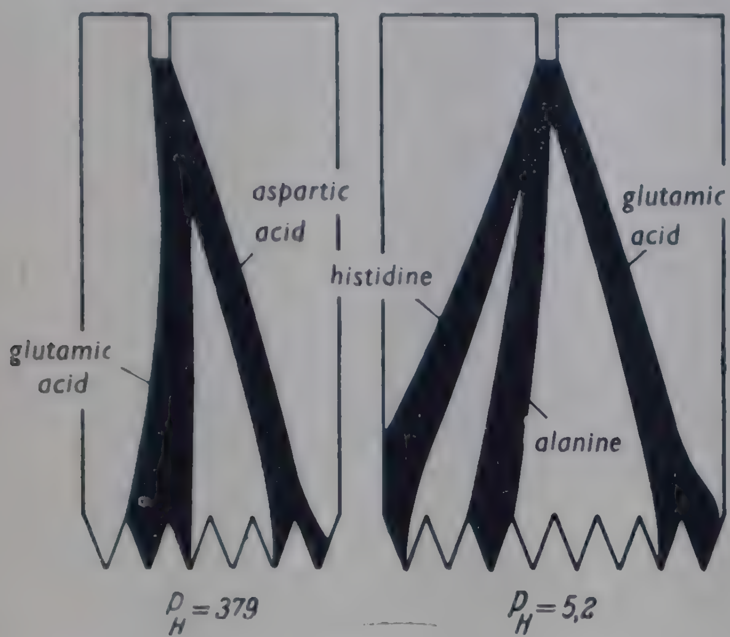


FIG. 54.—Continuous paper electro-pherogram (132).

between two platinum electrodes in a frame of plexiglass. In order to prevent fluctuations in the concentration of the buffer at the electrodes, an intensive current of the same buffer solution is maintained over their surfaces by laying thick strips of wadding over them which dip into the same bath of buffer solution as the upper end of the filter-paper. Duration of experiment 2–6 hr. Tension 110 V.

After developing the paper, the streaks leading to the various vessels at the base may be clearly seen, and thus the distribution of substances among these is apparent. The substances separated in this manner may then be isolated from the respective collecting vessels.

Similar pieces of apparatus have also been suggested by other workers (275, 101, 295).

II. SPECIAL SECTION

Amino-acids

It was in the separation of amino-acids that the technique of paper chromatography first found application. This separation is of particular importance, since no suitable micro-method was hithero known for separating the thirty-odd fundamental protein units.

1. Protein hydrolysis

Paper chromatographic studies with amino-acids generally arise in connection with the analysis of the fundamental units of some protein. The protein in question must therefore first be hydrolysed. The simple method described by Zahn (357), which has stood the test of time in the institute here, is given below:

50 mg. protein (polypeptide, peptide, etc.) are heated together with 0·5 c.c. 6*N* hydrochloric acid in a sealed glass tube to 110° C. in a drying-oven for 24 hr. After cooling, the tube is opened up and the contents are poured out into a test-tube 3 × 20 cm. The tube is then closed with a bung fitted with a glass tube and, if necessary, some anti-spray device, and evacuated so as to remove water and hydrochloric acid. To facilitate evaporation, the tube should be held in a boiling water-bath with gentle shaking. After evaporating to dryness, air is drawn for $1\frac{1}{2}$ hr. through the test-tube maintained at 100°. This ensures almost complete removal of the hydrochloric acid. The dry residue is taken up in 2·5 c.c. water or 10% *iso*-propanol so that the concentration of the hydrolysed product is about 2%. Should the solution still give an acid reaction with p_H paper, it should be cautiously treated with one drop of *N*/10 caustic soda.

2–10 mm.^{3} of this solution—the amount depending on the number of amino-acids expected—are then applied to the paper.

2. Solvents

In addition to the common ones—phenol, butanol–acetic acid and collidine—the following may also be used: furfural, tetrahydrofuran (19), *n*-propanol–water (7 : 3) *tert.*-butanol-methyl ethyl ketone–water (4 : 4 : 2), *tert.*-butanol–methanol–water (4 : 5 : 1) (30), benzyl alcohol, amyl alcohol, picoline, glacial acetic acid–pyridine–water (35 : 50 : 15), methanol–NH_3–water (90 : 2 conc. : 8), *iso*propanol–water–NH_3 (80 : 18 : 2 conc.) (85), *iso*-butanol-2N-acetic acid (400 : 75), *iso*butyric acid–water (80 : 20) (354). The choice of a suitable solvent will depend in each case on the particular mixture of amino-acids to be separated, and may be varied almost infinitely.

In the two-dimensional method, phenol is used in one direction and collidine in the other (68), or the pair *tert.*-butanol–methyl ethyl ketone–water and *tert.*-butanol–methanol–water for the separation of the following

ubstances: alanine, glycine, *iso*leucine, leucine, methionine, phenylamine, roline, threonine, tryptohane, tryosine, valine. The solvent pair phenol–vater and *n*-propanol–water may be employed for the separation of aspartic cid, glutamic acid, alanine, arginine, asparagine, cystine, glycine, histidine, *so*leucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine. Working with an ascending two-dimensional chromatogram and the last-named solvent pair, it is possible to differentiate all amino-acids (29). Leucine and *iso*leucine, which are otherwise difficult to separate, may be parted with amyl alcohol in a continuous running chromatogram (216, 349), or with methyl ethyl ketone 70, pyridine 15 and water 15 (337).

The following are recommended as standard solvents for two-dimensional paper chromatography:

In one direction:
  80% aqueous phenol

In the other direction:
  either collidine, see p. 19,
  or 75 parts *sec.*-butanol, 15 parts formic acid (88%), 10 parts water. The butanol–acetic acid mixture described on p. 19 has very similar properties.

The key B and the coloured plate I are intended for these solvents. Fig. 55 (see folder facing p. 46 and coloured plate I) shows a map of amino-acids obtained as described in (92).

3. Developing

As the solvent evaporates off, fluorescent compounds are formed between the cellulose and the amino-acids on warming (247, 346). Only tryptophane, histidine and citrulline are self-fluorescent. The fluorescence, however, depends to a very marked extent on the nature of the paper. If it is desired to wash out peptides after running a chromatogram, this should be heated to 100° for 20 min., after which the patches may be recognized in ultra-violet light.

The ninhydrin reaction: spray with a 0·2% ninhydrin solution in 95% butanol and 5% 2*N* acetic acid, subsequently warming to 105° until the spots become clearly visible. A gentler treatment, which has more to recommend it, consists in suspending for 24 hr. at room temperature. The spots are stable for a few days, but begin to lose their sharp contours after 36 hr. A positive ninhydrin reaction is sometimes given by peptides and proteins.

The ninhydrin coloration may be rendered permanent in the following manner (173):

0·2 c.c. 10% nitric acid is added to 1 c.c. saturated copper nitrate solution and the mixture is made up to 100 c.c. with 95% methanol. The ninhydrin

spots are then sprayed with this copper nitrate solution. The copper complex thus formed is very durable, especially if the chromatogram is exposed to ammonia immediately after spraying. Peptides may be developed with hydrogen chloride or chlorine gas (*v.* Perlon).

If it is desired to wash out the amino-acids for a subsequent quantitative estimation, the following procedure may be employed (253):

The paper is sprayed with a 0·1% solution of orcine in 0·004*N* sulphuric acid and afterwards heated to 110–120° for half an hour. The amino-acids on the paper act as a buffer and prevent the acid attacking the cellulose at these points, while the cellulose on the rest of the paper is partially hydrolysed and the sugar formed produces a red-violet coloration with the orcine. White areas on a coloured background are thus obtained, and these may now be cut out. It should be remembered, however, that prolonged application of heat causes considerable injury to the amino-acids (50).

Other developers: As a universal reagent iodine gives light yellow or brown spots (39). The amino-acids are not destroyed in the process and may afterwards still be estimated quantitatively. *o*-Phthalaldehyde produces a green spot with glycine (243).

The optical configuration of the amino-acids may be ascertained by spraying with *d*-amino-acid oxidase (299). *d*-Amino-acids are thereby destroyed, and thus no longer give a positive ninhydrin reaction, while *l*-amino-acids withstand the operation unchanged.

Histidine produces a hardly recognizable spot with ninhydrin, and this amino-acid is therefore best developed with diazotized sulphanilic acid, which is also suitable for tyrosine. The method of carrying this out is treated under development of phenols.

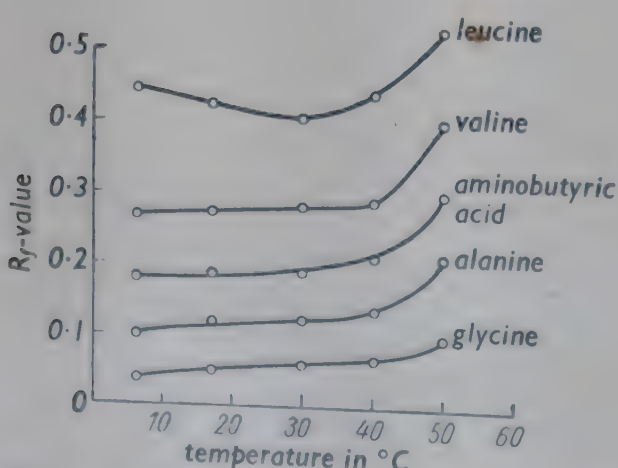


FIG. 56.—Variation of R_f-value with temperature (169) in 90% aqueous propanol.

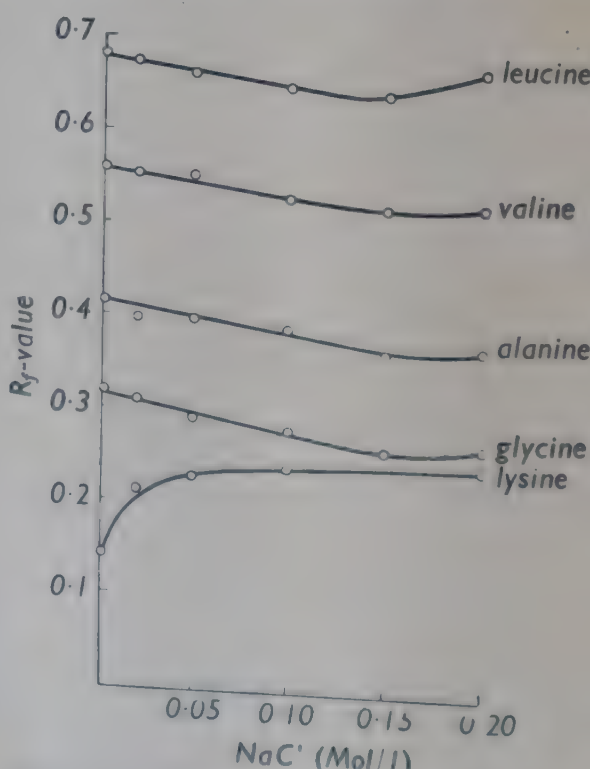


FIG. 57.—Variation of R_f-value with salt content of solvent (169) in 68% propanol, 32% water.

Proline and hydroxyproline yield only light yellow spots with ninhydrin. It is therefore better to spray with a solution of 0·3% isatin in *n*-butanol containing 4% acetic acid (1a).

Tryptophane derivatives also give a very weak ninhydrin reaction. These are therefore sprayed with Ehrlich's reagent: 2 g. dimethylaminobenzaldehyde in 100 c.c. 20% hydrochloric acid, and developed at 60°.

The colour reaction with cinnamic aldehyde has also proved useful here (337):

The chromatogram is sprayed with a 1% solution of freshly distilled cinnamic aldehyde in methanol, and after evaporation of the methanol, is brought into a concentrated atmosphere of HCl gas. Tryptophane—dark brown-red proline and hydroxyproline—pale brown-violet, hydroxytryptophane—yellow.

The variation of the R_f-values of amino-acids with changes of temperature and salt concentration are shown in Fig. 56 and 57 (169).

Table of R_f*-values (68).*

The R_f values in this table refer to Whatman No. 1 paper unless otherwise indicated.

	Phenol HCN	Phenol 3% NH_3	Phenol 0·1% NH_3	Phenol coal gas	Phenol cupron	Collidine	*n*-Butanol 3% NH_3	*tert.*-Amyl alcohol	Benzyl alcohol	Butanol–benzyl alcohol. 1 : 1-HCN	*o*-Cresol
Glycine	0·40	0·40	0·41	0·40	0·42	0·25	0·05	0·07	0·02	0·03	0·07
Alanine	0·54	0·54	0·55	0·57	0·59	0·32	0·09	0·10	0·03	0·05	0·13
Norvaline	0·81	0·79	0·78	0·78	0·80	0·48	0·31	0·23	0·12	0·19	0·45
Valine	0·77	0·76	0·76	0·78	0·77	0·45	0·22	0·18	0·11	0·15	0·41
Norleucine	0·88	0·85	0·87	0·85	0·89	0·60	0·51	0·42	0·27	0·36	0·72
*iso*Leucine	0·86	0·81	0·87	0·82	0·86	0·54	0·40	0·31	0·18	0·27	0·58
Leucine	0·85	0·83	0·86	0·84	0·88	0·58	0·46	0·36	0·21	0·31	0·61
Phenylalanine	0·89	0·87	0·90	0·96	0·93	0·59	0·46	0·36	0·36	0·38	0·81
Tyrosine	0·64	0·63	0·66	0·59	0·62	0·64	0·14	0·24	0·14	0·19	0·24
Serine	0·36	0·33	0·34	0·33	0·36	0·28	0·05	0·08	0·01	0·02	0·05
Threonine	0·50	0·41	0·47	0·50	0·50	0·32	0·08	0·09	0·02	0·04	0·11
Oxyproline	0·67	0·50	0·66	0·66	0·67	0·34	0·05	0·10	0·04	0·05	0·27
Tryptophane	0·83	—	—	0·76	0·86	0·62	—	—	—	0·30	0·65
Histidine	0·69	0·68	0·70	0·72	0·69	0·28	0·09	0·07	0·02	0·03	0·25
Arginine	0·59	0·89	0·85	0·67	0·62	0·16	0·05	0·02	0·01	0·01	0·02
Ornithine	0·33	0·73	0·61	0·40	0·37	0·13	0·03	0·02	0·00	0·00	0·01
Lysine	0·46	0·82	0·73	0·50	0·48	0·14	0·02	0·02	0·00	0·01	0·01
Aspartic acid	0·15	0·12	0·12	0·14	0·17	0·22	0·01	0·01	0·02	0·00	0·00
Glutamic acid	0·25	0·13	0·19	0·24	0·28	0·25	0·01	0·02	0·00	0·01	0·01
Lanthionine	0·20	0·19	0·21	0·18	0·20	0·12	0·00	0·03	0·00	0·00	0·01
Cystine	0·30	0·24	0·13	0·13	0·29	0·14	0·01	0·02	0·00	0·00	0·02
Methionine	0·90	0·76	0·83	0·82	0·81	0·57	0·05	0·27	0·17	0·21	0·58
Anserine	0·92	—	—	—	—	0·10	—	—	—	—	—
Carnosine	0·83	—	—	—	—	0·09	—	—	—	—	—
Creatine	0·82	—	—	—	—	—	—	—	—	—	—
Creatinine	0·94	—	—	—	—	—	—	—	—	—	—

R$_f$-*values of Amino-acids in Solvents Miscible with Water* (*19*)

Solvent	Tetrahydro-furan	Tetrahydro-fufuryl alcohol	Pyridine	Acetone	Acetone	Furfuryl-alcohol	Furfuryl-alcohol	Furfuryl-alcohol	Furfuryl-alcohol
Vol. % water	40%	20%	35%	40%	40%+0·5% Urea	25%	25%+0·5% Urea	25%+8% Pyridine	25%+5% Furan-α-carboxylic acid
Whatman no.	1	4	1	1	1	4	4	4	4
Direction of running	Ascending	Descending	Ascending	Ascending	Ascending	Descending	Descending	Descending	Descending
Aspartic acid	0·38	0·15	0·43 B.	0·45	0·38	0·18	0·20 B.	0·07	0·23
Glutamic acid	0·45	0·19	0·48 P.	0·51	0·46	0·18	0·23 P.	0·09	0·33
Cystine	—	0·20	—	—	—	0·16	0·19 G.	0·06	0·11
Serine	0·48	0·34	0·51 P.	0·47	0·44	0·20	0·29 P.	0·14	0·26
Glycine	0·44	0·28	0·41 R.P.	0·45	0·42	0·21	0·28 Br.	0·15	0·29
Ornithine	0·30	0·31	0·29 P.	0·16	0·19	0·24	0·28 P.	0·10	0·24
Arginine	0·34	0·27	0·31 P.	0·15	0·20	0·34	0·32 P.	0·17	0·32
Histidine	0·59	0·31	0·43 G.P.	0·38	0·33	0·25	0·39 Br.	0·22	0·31
Lysine	—	—	—	—	—	0·25	0·20 P.	0·13	0·28
Alanine	0·67	0·43	0·54 P.	0·55	0·52	0·34	0·39 P.B.	0·26	0·47
Threonine	0·66	0·46	0·56 P.	0·57	0·50	0·35	0·34 P.	0·22	0·37
Oxyproline	0·63	0·39	0·56 G.	0·53	0·54	0·35	0·41 G.	0·32	0·43
Tyrosine	0·72	0·59	0·65 G.P.	0·63	0·69	0·46	0·47 G.	—	0·52
Valine	0·73	0·64	0·65 P.	0·62	0·65	0·50	0·52 B.P.	0·43	0·64
Methionine	0·66	0·67	0·66 P.	0·63	0·65	0·55	0·58 P.	0·42	0·59
Proline	0·73	0·49	0·56 G.	0·58	0·62	0·56	0·56 G.	0·42	0·61
Tryptophane	0·78	0·70	0·63 Br.	0·58	0·64	0·56	0·59 G.P.	0·43	0·61
Leucine	0·78	0·72	0·68 P.	0·69	0·71	0·59	0·63 P.	0·43	0·69
*iso*Leucine	0·78	0·71	0·66 P.	0·67	0·71	0·59	0·63 P.	0·43	0·70
Phenylalanine	0·79	0·73	0·66 P.	0·64	0·67	0·63	0·64 G.P.	0·52	0·72

Coloration with ninhydrin: B. = blue, Br. = brown, P. = purple, G. = yellow, R.P. = red-purple, G.P. = grey-purple, B.P. = blue-purple. Unless otherwise indicated, the colours are the same as in pyridine.

R_f-*values of Amino-acids* (20)

Phen = phenol saturated with an aqueous solution of 6·3% sodium citrate and 3·7% prim. phosphate. BuAc = butanol 40, glacial acetic acid 10, water 10.

Compound	Phen	BuAc	Compound	Phen	BuAc
Alanine	0·55	0·38	Homoserine	0·47	0·30
β-Alanine	0·55	0·37	Oxyproline	0·59	0·30
α-Aminobutyric acid	0·65	0·45	*iso*Leucine	0·79	0·72
γ-Aminobutyric acid	0·78	0·50	Kynurenine	0·43	0·76
α-Aminocaprylic acid	0·74	0·85	Leucine	0·79	0·73
α-Amino*iso*butyric acid	0·68	0·48	Lysine	0·39	0·14
Arginine	0·41	0·20	Methionine	0·73	0·55
Asparagine	0·29	0·19	Methionine sulphone	0·53	0·28
Aspartic acid	0·07	0·24	Methionine sulphoxide	0·72	0·25
Citrulline	0·56	0·25	Norleucine	0·84	0·74
Cysteine	0·19 0·57	0·07 —	Norvaline	0·73	0·65
Cystine	0·08	0·08	Ornithine	0·27	0·15
αγ-Diaminobutyric acid	0·25	0·12	Phenylalanine	0·78	0·68
Dioxyphenylalanine	0·30	0·24	Proline	0·85	0·43
Diiodotyrosine	0·80	0·70	Serine	0·24	0·27
Glutamic acid	0·16	0·30	Taurine	0·29	0·19
Glutathione	0·25	0·05	Threonine	0·39	0·35
Glycine	0·30	0·26	Trimethylalanine (Neo-leucine)	0·81	0·66
Glycylglycine	0·39	0·22	Tryptamine	0·85	0·73
Hippuric acid	0·75	0·93	Tryptophane	0·66	0·50
Histamine	0·52	0·22	Tyrosine	0·52	0·45
Histidine	0·55	0·20	Valine	0·64	0·60

R_f-*values of Amino-acids in Solvents Containing Water* (85)

	α-Picoline	s-Collidine	Collidine	Glacial acetic acid Pyridine	Methanol	*iso*Propanol	Phenol
Glycine	0·27	0·25	0·14	0·41	0·38	0·26	0·40
Alanine	0·40	0·32	0·19	0·57	0·51	0·36	0·55
Aminobutyric acid	0·47	—	0·23	0·64	0·62	—	—
Valine	0·55	0·45	0·30	0·70	0·62	0·53	0·78
Leucine	0·60	0·58	0·32	0·72	0·67	0·57	0·84
*iso*Leucine	0·62	0·54	0·35	0·65	0·66	0·63	0·82
Proline	0·43	0·35	0·25	0·55	0·52	—	0·87
Oxyproline	0·39	0·34	0·24	0·44	0·44	0·31	0·66
Serine	—	0·28	—	—	0·43	—	0·33
Cystine	0·17	—	0·11	0·20	0·28	0·08	—
Methonine	0·59	0·57	0·42	0·70	0·56	0·47	0·82
Phenylalanine	0·67	0·59	0·55	0·73	0·56	0·61	0·86
Tyrosine	0·63	0·64	0·41	0·65	0·49	0·30	0·59
Tryptophane	0·64	0·62	0·50	0·67	0·42	0·39	0·76
Lysine	0·14	0·14	0·06	0·35	0·36	0·15	0·50
Arginine	0·20	0·16	0·11	0·43	0·34	—	0·67
Histidine	0·31	0·28	0·18	0·37	0·37	0·19	0·72
Glutamic acid	0·14	0·25	0·14	0·47	0·34	0·15	0·24
Aspartic acid	0·22	0·22	0·09	0·29	0·42	0·13	0·14

R_f*-values of Tryptophane Derivatives* (*147*)

	Phenol-NH_3	Butanol–acetic acid	Collidine
Tryptophane	0·78	0·50	0·62
Oxytryptophane	0·84	0·48	0·60
Kynurenine	0·80	0·45	0·55
3-Hydroxykynurenine	0·63	0·40	0·65

4. Quantitative estimation

This is possible on the paper chromatogram:

1. From the size and intensity of the spots (44, 115, 252). With the aid of a suitable photometric arrangement, it is possible to make direct readings of the intensity (27, 52, 121, 244, 267, 301).

2. The soluble copper complexes of amino-acids may be estimated with diethyl thiocarbaminate (347).

3. Polarimetric estimation of copper complexes (211).

4. Micro–Kjeldahl estimation (180).

5. The substances are transformed into their N-*p*-^{131}I-phenylsulphonic acid derivatives, after which the radio-activity of the spots may be measured. If separation is incomplete, a known quantity of the N-^{35}S-*p*-iodosulphonic acid derivative is added to the mixture, thus marking the spot being sought, after which the amino-acid in question may be estimated from the ratio $^{131}I : ^{35}S$ (175, 176).

6. Colorimetric estimation with ninhydrin. Numerous modifications of this have been described (9, 222, 230, 252). The following procedure would appear to be among the best (31):

Reagents:

1. 1% solution KOH in anhydrous methanol.
2. Solution of ninhydrin hydrindantin of p_H–4·7: 5 g. ninhydrin and 500 mg. $SnCl_2$, $2H_2O$ are dissolved in a mixture of 500 c.c. methyl cellosolve (= glycol monomethyl ether), 250 c.c. 1*N* NaOH and 250 c.c. 2*N* acetic acid. The acetic acid solution should be prepared from glacial acetic acid which has been treated with chromic acid and distilled in order to remove traces of ammonia. The solution may be stored for 24 hr. in a stoppered bottle and for several weeks in an atmosphere of nitrogen.
3. Solution for dilution: *n*-propanol–water 1 : 1.

Removal of ammonium ions:

After the spots have been rendered visible with a wire brush, a pencil line is drawn round each one, leaving a 1-cm. margin. The paper is then sprayed with the methanolic potash and maintained at 60° for 15 min. As a result of this treatment, the ammonium ions are expelled.

Estimation of the amino-acids:

The spots are cut out by cutting round on the inside of the pencil lines. The little pieces of paper obtained in this way are introduced into separate test-tubes, 1·5 × 16 cm., and treated with the ninhydrin reagent. The test-tubes are now maintained for 20 min. in a bath of boiling water and then immediately transferred

to cold water. 5 c.c. of the dilution solution are added to each tube and the mixture is shaken. After 10 min., but before 2 hr., the extinction coefficients against water are measured at 570 mμ (for proline 440 mμ). The 'blind' values must be subtracted. The 'blind' value of Whatman paper (1), which has been treated with alcoholic potash, is set equal to 0. The method allows of an accuracy of at least ± 5%. The components (up to 18) of a mixture of 0·2–0·3 mg. hydrolysed protein may be estimated in this way.

The somewhat troublesome use of the wire brush may be avoided if the chromatogram be heated for 15 min. at 100°. After this treatment the amino-acid spots may, as already stated, be recognized by their blue fluorescence. They can then be extracted and estimated colorimetrically with ninhydrin (121a).

Factors for the Estimation of Amino-acids Colorimetrically (31)

Amino-acids	Extinction coeff. for 0·1 mol amino-acid at 570 mμ	Leucin factor = Extinction coeff. for mol amino-acid at 570 mμ for leucin = 1
Aspartic acid	0·189	0·93
Glutamic acid	0·190	0·93
Alanine	0·206 ✓	1·01
Arginine	0·221	1·08
Cystine	0·222	1·04
Glycine	0·204	1·00
Histidine	0·193	0·95
*iso*Leucine	0·205	1·00
Leucine	0·204	1·00
Lysine	0·222	1·09
Methionine	0·204	1·00
Phenylalanine	0·192	0·94
Proline	0·012 (0·052 at 440 mμ)	0·06 (0·26 at 440 mμ)
Serine	0·194	0·95
Threonine	0·191	0·94
Tryptophane	0·149	0·73
Tryosine	0·183	0·90
Valine	0·203	1·00

7. Retention analysis, see p. 35.

For paper chromatography with columns of filter-paper, see p. 37.

For partition chromatography of amino-acids on starch, the original literature should be consulted (221, 224).

5. A selection of results

taken from the large number of experiments of current interest are added to demonstrate the extraordinary versatility of the method and to give some idea of the advances made possible by it.

(*a*) *Uncommon or new amino-acids.* α-Amino-ββ-dimethyl-γ-hydroxy-butyric acid probably occurs in *E. coli* (1). α-Amino-adipic acid may be separated from glutamic acid with *n*-butyric acid—*iso*valeric acid (21). Arginine is formed in the liver out of this and citrulline (97). γ-Amino-butyric acid occurs in yeast and other biological material (10, 258, 291, 349).

N-alkylamino acids can be recognized on the chromatogram by virtue of th fluorescence (248). α-Amino-ε-aminopimelic acid is found in certain bacte (350).

The paper chromatographic study of the formation of thyroxine organisms is of great interest (67, 151, 300, 303). As already mentioned, t may either be identified with radio-active iodine or in the normal mann with ninhydrin.

The decomposition products of tryptophane may be worked up qu simply by a paper chromatographic method (214).

(*b*) *Free amino-acids in animal and vegetable tissues.* It is only propos to deal with a few typical examples of the manifold application to the inves gation of the amino-acid content of natural fluids. The amino-acid conte of urine under normal and pathological conditions has been the subject numerous investigations (4, 89, 90, 137). The amino-acid content of tumou (60, 264), cellular fluids (2, 88, 264) and of egg (196) have been investigate The most important results, however, were obtained by a paper chromat graphic study of the

(*c*) *Constitution of peptides and proteins.* Wool contains twenty-o different amino-acids (69, 354) and numerous peptide fractions (71, 71a On boiling with water, the so-called wool-gelatine is extracted, which was t subject of at least one investigation (354). The epicuticula of wool was al investigated (359). The fermentative degradation of wool may readily followed on the paper chromatogram (24). Both the qualitative and quantit tive constitution of clupein may be ascertained (111). Numerous investig tions on fibre proteins—e.g. (149). On nitrating silk, the hydrolysed produ contains 3-nitro- and 3 : 5-dinitrotyrosine having $R_{val.}$-values of 0·7 and 0·14 respectively in *iso*butanol–acetic acid–glycol–water (355). A larg number of specific peptides and proteins have been investigated. Thu gramicidine contains *d*-leucylglycine, *l*-alanyl-*d*-valine and *l*-alanyl-*d*-leucin in its peptide ring (70a, 143, 299). The *d*-amino-acids were elucidated by th fermentative method mentioned above. The poisonous principles *Amanita vulgaris*, amanithine and phaloidine also possess a cyclic structu (331). The constitution of the amino-acids in the snake poison crotoxin has been elucidated (285).

The products of hydrolysis of nylon, perlon and polyurethane may b identified on the paper chromatogram (356, 358).

According to a process devised by Zahn, the polyamides themselves are rendere visible by immersing in a mixture consisting of equal parts chloride water and N/1 hydrochloric acid, as a result of which the respective chloramides are formed. O washing with water and dipping the paper first into potassium iodide and then into starc solution, the polyamides may be located by the blue-green coloration which they giv This procedure is also well suited for developing peptides. As solvent *sec*butanol formic acid–water or 60% propanol, 30% ammonia, 10% water may be employed. O spraying with a 0·04% bromothymol-blue solution of p_H 10, dicarboxylic acids giv yellow spots and hexamethylenediamine blue ones.

(*d*) The DNP (= dinitrophenyl) derivatives of the amino-acids, which are now widely employed for fixing protein end-groups (269), may be run on a paper chromatograph after hydrolysis (23, 217, 220, 242). For this purpose, the paper must be buffered. Since the DNP derivatives are more lipophilic than the free amino-acids, they migrate rather rapidly on filter-paper, and there is now a growing tendency to separate them on filter-paper columns which have been impregnated with caoutchouc (cf. p. 100). This renders the organic phase stationary, and the DNP derivatives now migrate slowly (241) (cf. p. 58).

(*e*) Peptides are easy to deal with on the paper chromatogram and may also be developed with ninhydrin (10, 35, 124) or chlorine water (356). It is also possible to check the progress of a peptide synthesis on a paper chromatogram (140, 142, 143, 256).

A definite relationship has been found to exist between the R_f-values and the thermodynamic data of peptides (212).

The paper chromatography of the peptides is of particular significance because it is possible, with its aid and the help of end-group estimation with dinitrofluorobenzene, to ascertain the order of the amino-acids in a protein. The protein is first partially hydrolysed, after which the various peptides are isolated on a paper chromatogram. The individual peptides are cut out, fully hydrolysed and their amino-acid constitution elucidated on a further paper chromatogram. An extension of this system leads to excellent results.

R_f*-values of the Products of Hydrolysis of Synth. Polyamides* (358)

Propanol/Ammonia	Whatman 1	
ε-Aminocaproic acid	0·61	Violet ninh.
Adipic acid	0·49	Yellow bromth.
AH-salt	0·50	Yellow bromth.
Hexamethylene diamine	0·78	Blue bromth.
Sebacic acid	0·72	Yellow bromth.
HS-salt	0·71	Yellow bromth.

R_f*-values of DNP-Amino-acids* (241)

Solvent	On filter-paper *tert.*-amyl alcohol	On allopren column *n*-butanol		
pH of buffer	7·75	5	4	3
DNP-Lysine	0·40	—	—	1
DNP-Asparagine	0·23	—	—	0·83
DNP-Serine	0·26	—	1	0·61
DNP-Aspartic acid	0·03	—	0·96	0·40
DNP-Glycine	0·27	—	0·79	0·33
DNP-Alanine	0·43	—	0·58	0·18
DNP-Proline	0·51	1	0·54	0·17
DNP-Valine	0·74	0·52	0·11	0·10
DNP-Leucine	0·86	0·35	0·09	0·08

R_f-*values of DNP-Amino-acids* (25)

Paper steeped in phosphate buffer $p_H = 6$.

	*cyclo*Hexane 30% propanol	*tert.*-Amyl alcohol	Benzyl alcohol 10% ethanol
DNP-Leucine	0·28	0·88	0·71
DNP-Valine	0·23	0·79	0·59
DNP-Phenylalanine	0·22	0·74	0·63
DNP-Alanine	0·15	0·46	0·36
DNP-Glycine	0·10	0·23	0·26
DNP-Threonine	0·07	0·36	0·26
DNP-Serine	0·05	0·21	0·18
DNP-Glutamic	0·05	0·04	0·07
DNP-Aspartic acid	0·02	0·00	0·03

R_f-*values of Peptides* (70)

Peptide	Phenol 0·1% cupron	*s*-Collidine	Butanol–acetic acid (73)	
Glycylglycine	0·53	0·28		
Glycyl-DL-alanine	0·64	0·32		0·37
Glycyl-DL-valine	0·78	0·47		0·37
Glycyl-DL-leucine	0·87	0·53		0·42
Glycyl-L-proline	0·77	0·34		
Glycyl-L-oxyproline	0·57	0·28		
Glycyl-L-phenylalanine	0·78	0·79		0·36
Glycyl-L-tryptophane	—	0·85	Diglycylphenylalanine	0·30
Glycyl-glycyl-glycine	0·58	0·32	Triglycylphenylalanine	0·23
Glycyl-glycyl-1-leucylglycine	0·86	0·71	Tetraglycylphenylalanine	0·18
DL-Alanylglycine	0·68	0·32	Pentaglycylphenylalanine	0·14
L-Alanylglycylglycine	0·67	0·46	Hexaglycylphenylalanine	0·11
DL-Valylglycine	0·83	0·53	Diglycylleucine	0·33
DL-Leucylglycine	0·86	0·61	Triglycylleucine	0·25
DL-Leucylleucine	0·95	0·89	Tetraglycylleucine	0·21
L-Leucyl-1-tryptophane	0·92	0·95	Pentaglycylleucine	0·15
L-Leucyl-glycylglycine	0·88	0·55		
L-Prolylglycine	0·86	0·49		
L-Tyrosylglycine	0·49	0·95		

The ascending series of glycyl peptides have the following R_f-value (46):

	Phenol	Butanol–acetic acid
Glycine	0·40	0·130
Glycylglycine	0·42	0·125
Diglycylglycine	0·47	0·096
Triglycylglycine	0·57	0·073
Tetraglycylglycine	0·67	0·053
Pentaglycylglycine	0·73	—

For further R_f-values of peptides, see (70a), (71), (71a), (273), (274).

Consden, Gordon and Martin (70) suggest the following technique:

The protein to be investigated is cautiously hydrolysed, as a result of which it is split up into peptides. The conditions chosen for hydrolysis depend entirely on the particular protein, and a preliminary test is necessary. The peptide mixture is now run in a pair of parallel two-dimensional paper chromatograms. One of these is developed with ninhydrin and used to locate the amino-acids on the second. The individual peptide spots located in this way are then cut out of the second chromatogram, although only the more intense blue spots will contain sufficient peptide for the following investigations. It is safer to develop the second chromatogram too, using a very dilute ninhydrin solution (0·01%) for this purpose. The single strips are washed into capillaries (cf. p. 35) and then transferred from the capillaries on to little plates of polyethylene. The drop is evaporated *in vacuo*. The amino-acid at the end of the peptide chain may now be destroyed with nitrous acid, for it is only this amino-acid which carries a free amino-group. The sample is therefore divided into two portions, one being deaminated, the other not. On finally investigating the amino-acids after complete hydrolysis, the amino-acid at the end of the chain will be missing in the deaminated sample.

Deamination:

The sample is dissolved in 30 mm.^{3} 6*N* hydrochloric acid on its polyethylene strip. The strip is pushed horizontally into a test-tube pre-warmed to 30–35°. Oxides of nitrogen, generated by dropping concentrated hydrochloric acid on to solid sodium nitrite, are led into the tube. Deamination is complete in 10 min., after which the strip is removed from the tube and dried over KOH *in vacuo*. The sample is again treated with hydrochloric acid and dried in order to remove any traces of oxides of nitrogen still present. The deaminated polypeptide is now submitted to hydrolysis.

Hydrolysis:

The sample is taken up in 20 mm.^{3} 6*N* hydrochloric acid. The solution obtained in this way is drawn up into a clean capillary, which is subsequently sealed at both ends. This is then maintained at 150° over-night. The next morning the contents of the capillary are brought on to polyethylene foil and dried *in vacuo* over KOH. The residue is then taken up in 5 mm.^{3} water. In order to avoid loss of substance, the solution is best applied directly to the starting point of the chromatogram.

The method has recently been refined, chiefly as a result of important work done by Sanger (272, 273, 274), who was in this manner able to go a long way towards elucidating the order of the amino-acids in insulin. As this work is of especial interest, both for the methods employed and in the results achieved, a detailed account of it is given here.

The insulin may be split up into its component polypeptide chains by oxidation of the S–S bonds. These can be separated because one fraction contains no basic amino-acids. By reaction with dinitrofluorobenzene (DNFB), it may be shown that the basic fraction contains a phenylalanine end-group (271). The order of the amino-acids in this phenylalanine chain was investigated. Among other experiments, the phenylalanine chain was submitted to partial hydrolysis by treatment with 11*N* hydrochloric acid at 37° for 4 days. After this the various peptide fractions were separated from one another with the aid of ionic exchangers and by adsorption on animal charcoal. In this manner five different main fractions were obtained. Paper chromatography was carried out with phenol in one direction and collidine or butanol–acetic acid in the other. After running, the chromatograms were dried and warmed to 100° for 20 min. A small fraction of the peptide spots is thereby transformed into a

fluorescent material, which may then be located under the quartz lamp. T fluorescence is, however, visible only in the complete absence of any traces of phen It is therefore essential to run in the first, and not the second, direction with phen The spots are pencilled off and, if clearly visible, cut out directly. Otherwise comparison must be made with a chromatogram run under the same conditions a developed, or the chromatogram may be developed with a very dilute ninhydrin so tion. The latter procedure should, however, be avoided if it is afterwards propos to carry out an end-group estimation with DNFB or by deamination, since it is w just these end-groups that the ninhydrin reacts.

Washing and final hydrolysis are performed exactly as described above, as is a the deamination.

The DNP-derivatives of the peptides are prepared by treatment with dinitr fluorobenzene in alkaline bicarbonate solution, extracting excess DNFB from t alkaline solution with ether. In order to free the DNP-peptides from accompanyi salts, they are extracted with ethyl acetate in acid solution or chromatographed talcum columns in acid or neutral solution. They are subsequently hydrolysed above for 20 hr., with 6*N* hydrochloric acid, the amino-acids obtained then bei estimated on a paper chromatogram. The DNP-amino-acids arising at the en groups are chromatographed using butanol–acetic acid and then have the followi R_f-values:

R_f-*values of DNP-Amino-acids in Butanol–Acetic Acid on Whatman 4 Paper.*

O-DNP-Tyrosine	0·84	im-DNP-Histidine	0·57
N-DNP-Arginine	0·81	DNP-Valine	0·49
N-DNP-Lysine	0·77	DNP-Cysteic acid	0·42
DNP-Leucine	0·67		

From a table of all di-, tri-, etc. peptides found, compiled after extremely laboriou micro-work, the following order of amino-acids in the complete molecule w established:

1. Phe-Val-Asp-Glu-His-Leu-$CySO_3H$-Gly.
2. Thr-Pro-Lys-Ala.
3. Gly-Glu-Arg-Gly-Tyr-Leu-Val-$CySO_3H$-Gly.
4. His-Leu-Val-Gly-Ala.

A complete arrangement of the amino-acids was possible with the aid of ferment tive hydrolysis. Larger degradation units may be obtained with the help of ferment which units are in turn compounded among themselves and with the peptides obtaine by acid degradation.

Enzymatic hydrolysis:

1. 30 mg. polypeptide are dissolved in 3 c.c. 0·01*N* HCl. This is treated with solution of 0·3 mg. pepsin (cryst.) in 0·01*N* HCl, and the mixture is maintained at 3 for 24 hr. After hydrolysis, the pepsin is inactivated by boiling, HCl is removed *vacuo*, the solution is purified by ionophoresis and worked up as after acid hydrolysi

2. 50 mg. insulin B fraction (= phenylalanine chain) are dissolved in 5 c.c. wat and brought to p_H 7·5 with bicarbonate. 2·3 mg. recryst. chymotrypsin are added this solution, which is then allowed to stand at 25° for 24 hr.

3. 25 mg. B fraction are dissolved in 2·5 c.c. water and brought to p_H 7·5. This treated with a solution of 1·2 mg. trypsin (cryst.) in 1·2 c.c. water and maintained 37° for 24 hr.

The B fraction of insulin thus possesses the following constitution:

Phe-val-asp(NH_3)-glu(NH_3)-his-leu-cys-gly-ser-his-leu-val-glu-ala-leu-tyr-leu val-cys-gly-glu-arg-gly-phe-phe-tyr-thre-pro-lys-ala.

It is only with the aid of paper chromatography that it has becom possible to solve problems of this type.

6. Proteins

The paper chromatography of proteins presents some difficulties.

Proteins show a marked tendency to denature and to become adsorbed, as a result of which a disturbing streaking occurs (122, 170, 283, 302, 351). Proteins may be dyed with wool dyes and their presence thus established. It was found possible to separate adenosine desaminase, amylase and a phosphatase on filter-paper columns using ammonium sulphate solution of various concentrations (219). This separation is, however, rather to be regarded as the result of the combined effects of adsorption, displacement and salting out.

The method employed for the separation of a selection of enzymes is to to be found in (127).

Identification of individual enzymes:

1. Amylases. 2 g. agar-agar, 1 g. soluble starch, 30 c.c. buffer p_H 4.6 (p_H 7 for amylase in saliva and *Aspergillus niger*) are made up to 100 c.c. and applied to a plate the size of the chromatogram. The chromatogram is then laid down on to the solidified agar-agar solution and left there for 4–12 hr. at room temperature. After this period it is cautiously raised and sprayed with 0·01*N* iodine solution. White or violet spots on a blue background.

2. Phosphatases. 0·1 g. Na-phenolphthalein phosphate and 2. g. agar-agar are dissolved in 30 c.c. phosphatases buffered to p_H 5·2 (p_H 9·2 for alkali) and made up to 100 c.c. This solution is spread on to a plate as above and covered with the chromatogram. On pouring *N*/10 caustic soda over the plate, rose-coloured spots on a colourless background make their appearance.

3. Phosphorylases. 0·2 g. di-potassium salt of glucose-1-phosphate is dissolved in 30 c.c. citrate buffer p_H 6·0, 2 g. agar-agar are added and the mixture is made up to 100 c.c. The chromatogram is laid on as before and sprayed with iodine, as a result of which blue spots due to amylase formed from the phosphorylase appear.

For solvents see table.

R_f-*values of Proteins at 0–5° C. on Whatman 1 (127)*

Ferment	Isolated from	Solvent	R_f
α-Amylase	*Aspergillus niger*	Acetone 50, water 50	0·75
β-Amylase	Potatoes	Acetone 50, water 50	0·50
α-Amylase	Saliva	Acetone 50, water 50	0·00
Amylases	Germinated rice	0·33 m. NaCl, acetone 75, water 25	0·00 and 0·67
Phosphorylase	Green gram	0·33 m. NaCl, or according to concentration	0·77–0·61
Acidic phosphatase	Rat's kidney	0·33 m. NaCl	0·61
Alkal. phosphatase	Rat's kidney	0·33 m. NaCl	0·00 and 0·67
Acidic phosphatase	Sheep's kidney	0·33 m. NaCl	0·53
Alkal. phosphatase	Sheep's kidney	0·33 m. NaCl	0·00 and 0·61
Acidic phosphatase	Sheep's kidney	Acetone 30, water 70	0·53
Alkal. phosphatase	Sheep's kidney	Acetone 30, water 70	0·00 and 0·60
Acidic phosphatase	Rat's liver	0·33 m. NaCl	0·63
Alkal. phosphatase	Rat's liver	0·33 m. NaCl	0·67
Alkal. phosphatase	Chicken serum	0·33 m. NaCl	0·65
Alkal. phosphatase	Human serum	0·33 m. NaCl	0·00
Alkal. phosphatase	Rat's serum	0·33 m. NaCl	0·00

Paper electrophoresis (see p. 39) is, however, considerably more suitab here, especially for the analysis of the serum proteins. A separation of fo ferments by paper electrophoresis is given in detail below as an examp (Wallenfels) (314). On applying paper electrophoresis to an extract fro suitable fungus cultures, amylase, proteinase, lipase and phosphatase ma be separated off.

25 mm.^{3} ferment solution are applied uniformly to a pencil line drawn across th middle of a strip of Whatman No. 4 filter-paper 10 cm. wide and 40 cm. long. Th solution thus forms a band 3 mm. wide in the middle of the paper. The paper brought to the desired p_H by spraying with a buffer solution of p_H 6·8 and the clamped in the electrophoresis chamber (327). The projecting ends are suspended

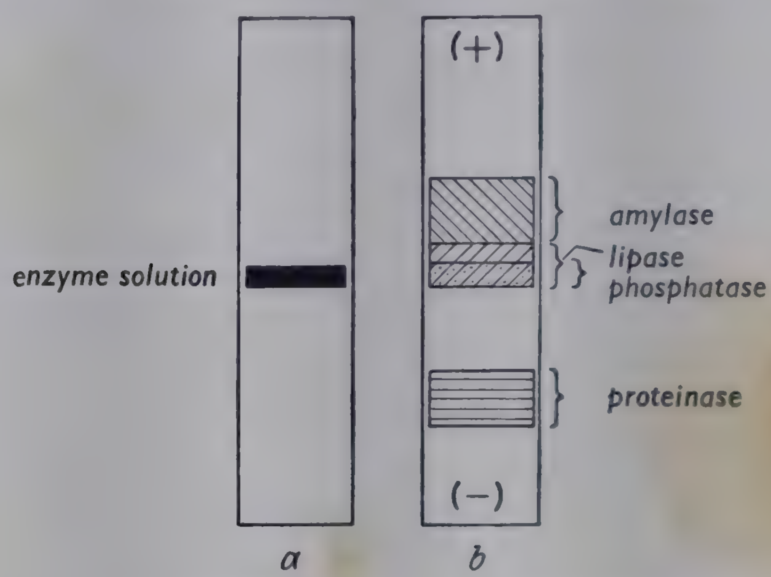
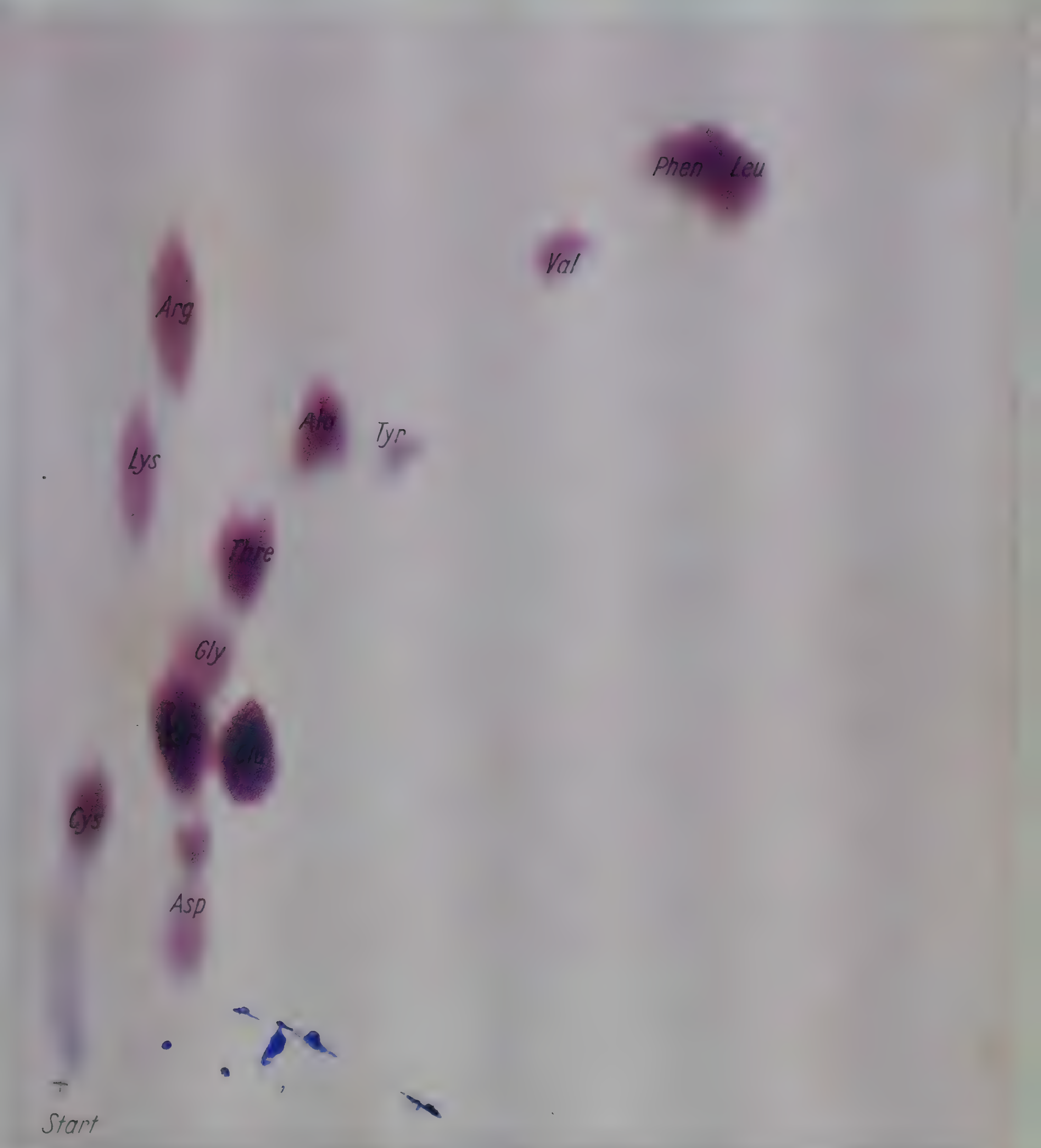


FIG. 58.—Paper electropherogram of a solution of 4 enzymes (314).

buffer solution of p_H 6·8. The electrodes are connected up to these solutions, so tha a tension of 120 volts may be applied across the paper. After passing the curren for 12 hr., the paper is removed and cautiously dried. The paper is now cut u longitudinally into strips 2 cm. wide, and single strips are employed for variou ferment tests.

1. The presence of proteinase may be demonstrated by its liquefying effect o gelatine. The strip is placed on a layer of swollen gelatine and maintained at 30° C
2. Amylase. Another strip is sprayed with starch solution, and then subjecte to the influence of the ferment at 40° C., subsequently being sprayed with a dilut solution of starch. The iodine–starch reaction fails to appear at the amylase spots
3. Lipase possesses the property of cleaving *p*-nitrophenylstearate, as a result o which a deep yellow band is formed on the paper.
4. Phosphatase may be identified in a similar manner with phenolphthalei phosphate.

It is not possible here to give a complete account of Tiselius's salting-out chromato graphy. The reader is referred to the original literature (302a).



75% sec. Butanol, 15% Formic acid, 10% Water ⟶

Two-dimensional chromatogram of a mixture of 10 amino-acids, developed with ninhydrin

(*Whatman* No. 1 paper)

By kind permission of Dr. *H. Zahn*

Plate II

Front

1 2 3 4

1 = Anthralangelb G (Höchst)
2 = Echtrot AV (BASF)
3 = Patentblau V extrakonz. (Höchst)
4 = Malachitgrün (Bayerwerk)
5 = Mixture: Anthralangrün GG Konz. } (Höchst)
Anthralanrot HG
Anthralangelb RRT

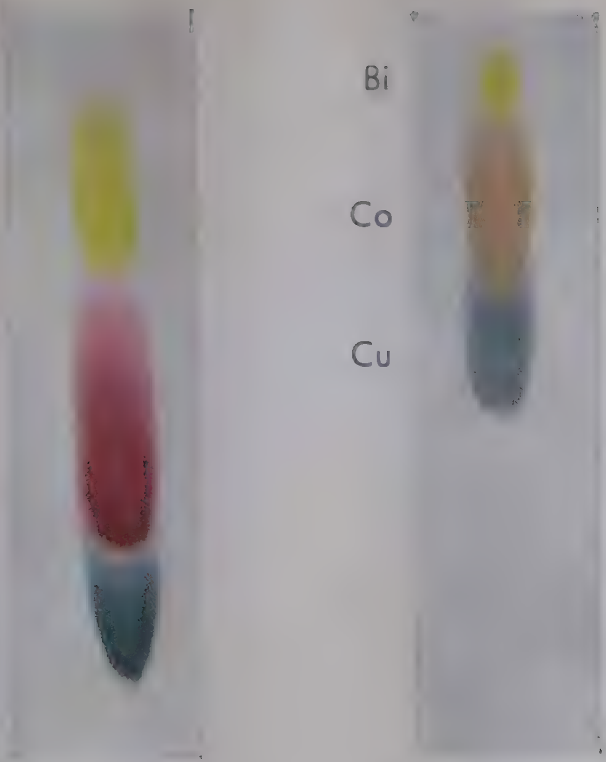
1% Solutions; 4 mm^3; 80% isobutyric acid.
(Paper: *Schleicher & Schüll* 2043 b)

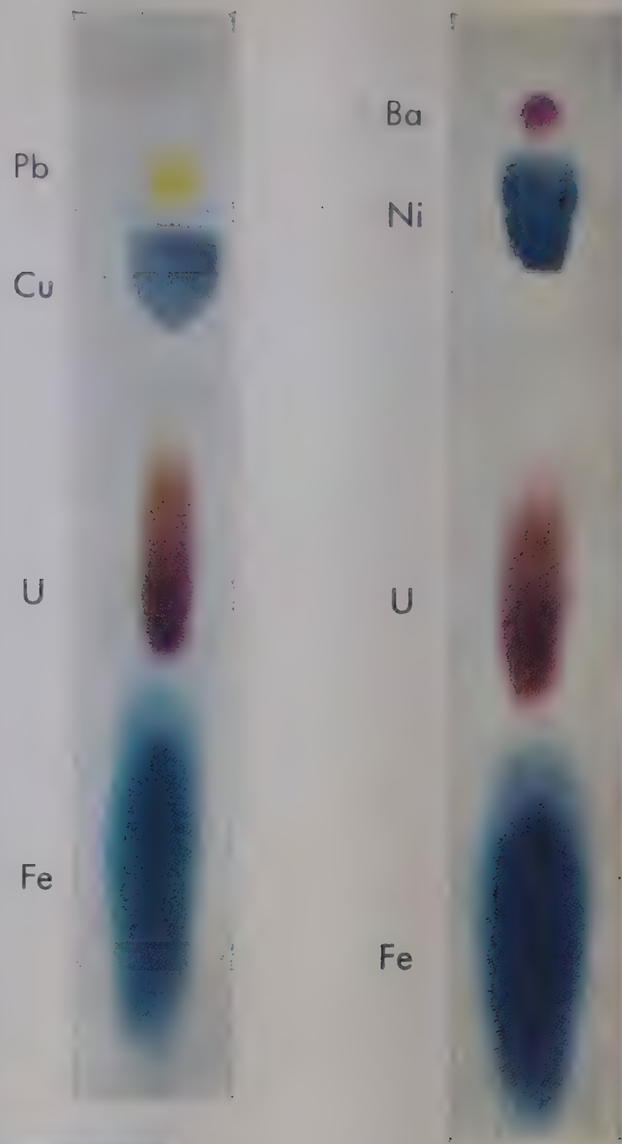
1 = Glucose
2 = Maltose
3 = Mixture
4 = Xylose
5 = Rhamnose

Start

1 2 3 4 5

One-dimensional chromatogram of a mixture of 4 sugars. Solvent, butanol-acetic acid, develop
with aniline phthalate.
(*Whatman* No. 1 pap





Separations achieved with the aid of paper chromatography generally exhibit well defined bands. The use of a complex forming compound in the mobile phase (e. g. benzoyl acetone in aqueous butyl alcohol) facilitates separation since the various metallic complexes probably have quite different coefficients in the partition between the aqueous and organic phases. The order obtained differs from that with water alone.

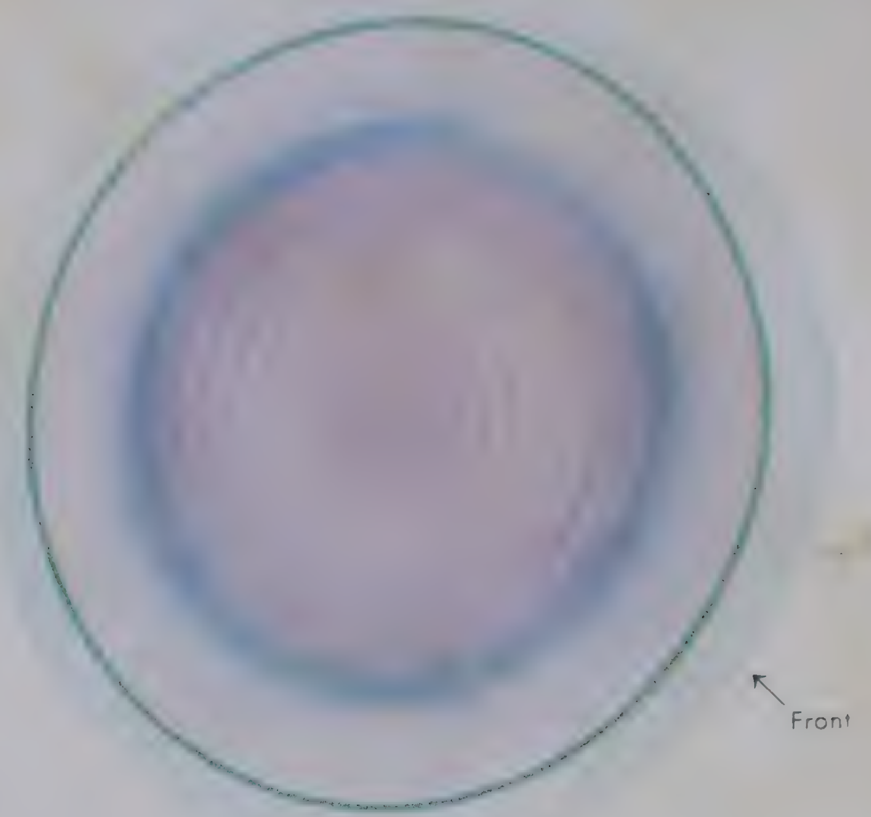
Ba

Pb

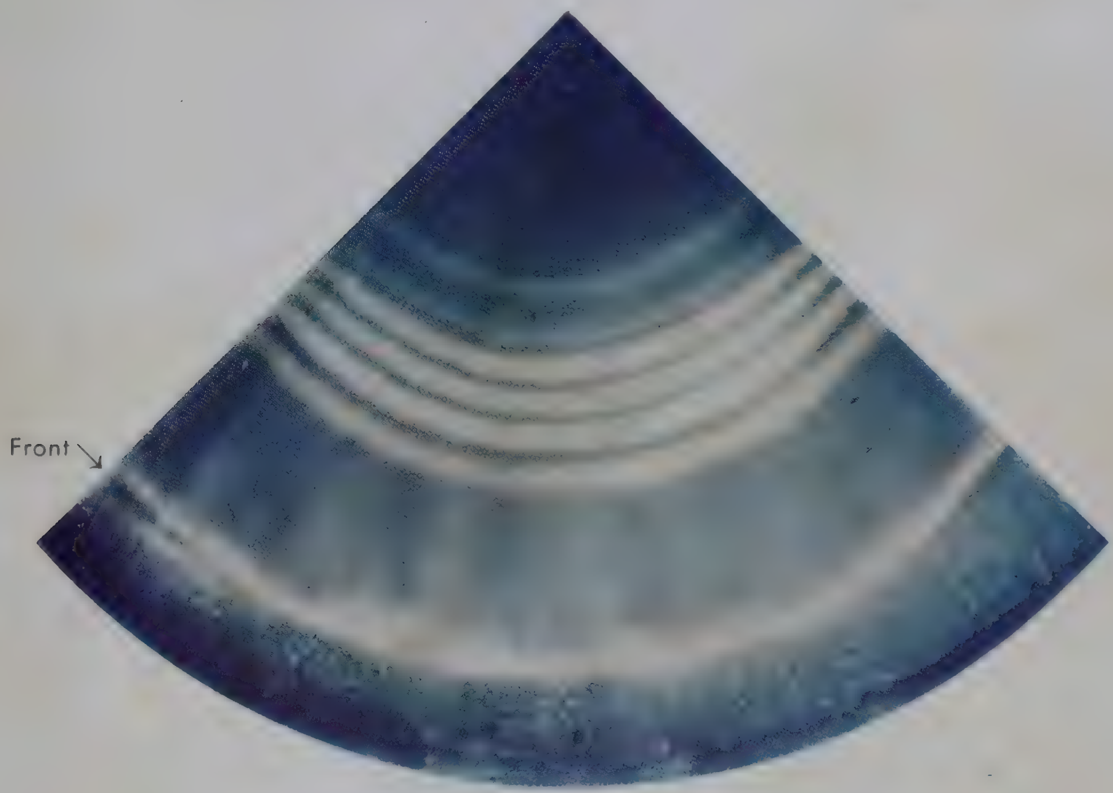
Cu

Two-dimensional separations often give improved results. The example shows a chromatogram run in one direction with an aqueous butyl alcohol solution containing benzoyl acetone, and in the other direction with an aqueous butyl alcohol solution containing trichloracetic acid.

Figs. in plate III taken from "Endeavour", vol. X, p. 213 (1951).



Circular chromatogram of Palatinechtmarineblau (BASF).
0,5 cc 0,5% qaueous solution. Solvent: 80% ethanol. 50 mins.
By kind permission of Dr. *H. Pfitzner*, BASF, Ludwigshafen



Circular chromatogram of the maltose homologues (78).
Solvent: butanol-dimethylformamide-water 2:1:1
Developed with aniline phthalate, photographed in uv-light
(Paper: *Schleicher & Schüll* 2045 b)

From outside to centre: Glucose, maltose, maltotriose, maltotetraose, maltopentaose (maltohexaose is only properly visible in the original), maltoheptaose. Hydrolysis product from cyclodextrine

Sugars

Before the discovery of the chromatographic process, the separation of sugars was almost more difficult than that of amino-acids. Paper chromatography has thus already become an almost indispensable tool of sugar chemistry and has rendered distinguished service (77, 237).

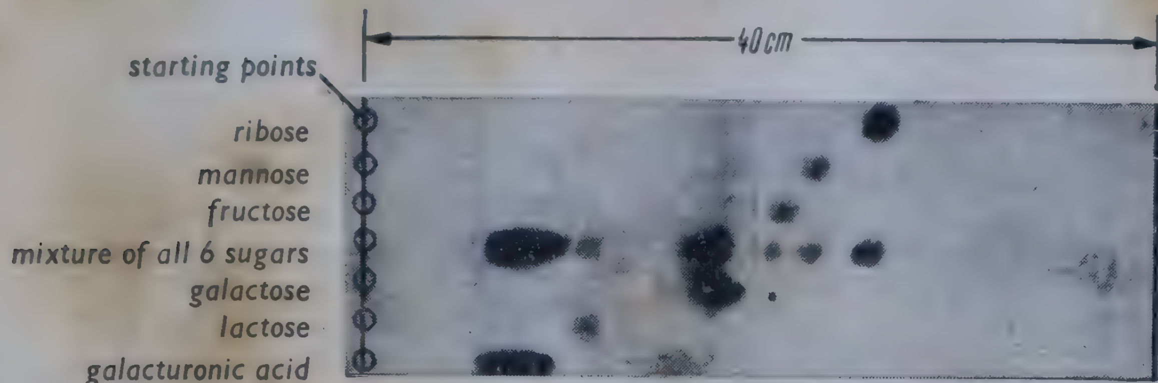


FIG. 59.—Paper chromatogram of a mixture of six sugars in which the individual sugars are also run simultaneously (237).

1. Solvents

Butanol–acetic acid, phenol–water, collidine, butanol–water. Butanol by itself yields very small R_f-values. Addition of acetic acid or acetic acid–glycol raises them quite considerably. Apart from these standard solvents, numerous other are, of course, possible. For the sake of simplicity it is, however, generally advisable to stick to a small number of standard combinations, provided these are found to give satisfactory results.

Phenol and butanol–acetic acid are employed when running in two dimensions.

2. Developing

(a) *Reducing sugars*:

1. Ammoniacal silver nitrate: The paper is sprayed with a mixture of equal parts of 0·1N silver nitrate and 5N NH_3 solution and developed at 105° for 5–10 min. (237). The sugars then appear as brown spots. If well-purified, freshly prepared solvents are not employed, silver is also deposited on the other parts of the paper, as a result of which the spots are hardly visible. This disadvantage governs the use of phenol. It is not possible to counteract the effect entirely by washing the dried chromatogram with ether (28). The addition of an equal volume of 2N NaOH to the silver nitrate reagent, with subsequent development over a steam bath, leads to somewhat better results.

2. Aniline phthalate (238). (The method of the author's choice.) 1·66 g. phthalic acid and 0·93 g. aniline are dissolved in 100 c.c. water saturated with butanol. The paper is sprayed with this mixture and warmed to 105° for

10 min. Aldopentoses produce reddish-brown spots with a red fluorescence other sugars olive-brown spots with a yellow fluorescence (cf. plates II and IV). The difference is especially characteristic under the uv-lamp. The method is very sensitive. Impurities in the paper or solvent have scarcely any effect. Fructose cannot be picked out quite as well as the other sugars, but its yellow fluorescent spot in visible under the uv-lamp without difficulty. The methylated sugars also give a characteristic colour reaction with aniline phthalate. Uronic acids also react readily.

3. *m*-Phenylenediamine forms acridine derivatives with sugars which are strongly fluorescent and thus constitute a sensitive test (66). The paper strip is sprinkled with an alcoholic solution of *m*-phenylenediamine dihydrochloride, warmed for a short time and then placed under the lamp. Glucose, mannose, galactose, fructose, sorbose, rhamnose and fucose all give yellow fluorescent spots; arabinose, xylose and ribose fluoresce with an orange colour.

4. Benzidine (157). 0·5 g. Benzidine, 20 c.c. glacial acetic acid, 80 c.c. abs. alcohol. Spray and heat to 105° for 15 min. Brown spots.

5. A number of other amines also give characteristic colour reactions—e.g. urea gives black spots with ketoses; *p*-anisidine. HCl in butanol (162) may be used in place of aniline phthalate, and yields similar results.

6. Triphenyl-tetrazolium chloride is suitable as a reagent for reducing sugars. The dried paper is sprayed with a freshly prepared mixture of 2% aqueous TTC solution and 1N caustic soda (1 : 1) and then suspended at 40° in an atmosphere saturated with water vapour. Excess TTC is cautiously washed out with water and the paper is dried at room temperature. The areas at which the sugars are concentrated now show up bright red. This reaction may be modified for a quantitative colorimetric estimation (313).

FIG. 60.—Paper chromatogram developed with triphenyltetrazolium chloride (313).

7. 3 : 4-Dinitrobenzoic acid (320). 1 g. dinitrobenzoic acid in 100 c.c. 2N soda solution. On heating to 100°, blue spots are formed which become brown on further heating or merely on standing. Ketoses react much more rapidly than aldoses, ascorbic acid and reductone react even at room temperature.

(b) *Non-reducing sugars*:

The presence of these on the paper may be demonstrated with the naphthoresorcinol test (120, 237), in which the sugars are dehydrated to fur-

fural derivatives by the action of acid. Furfural and its derivatives exhibit a marked analogy to the phenols and give characteristic and sensitive colour reactions. Care must, however, be exercised in carrying out the necessary treatment with acid sufficiently mildly to avoid attack of the cellulose in the paper. Trichloracetic acid is therefore the reagent best suited, since this splits off water to the desired extent, at the same time leaving the cellulose intact. For the colour reaction the paper is sprayed with a mixture of equal volumes of 0·2% alcoholic naphthoresorcinol solution and 2% aqueous trichloracetic acid and then heated to 100°. The reaction is specific for ketoses. Fructose, sorbose and cane-sugar produce brilliant red spots which are stable for 12 hr. If the paper is subsequently treated with water vapour at 70°, pentoses and uronic acids become visible as blue spots.

Glucosamine and *N* acetylglucosamine may be identified by the colour reaction of Morgan and Elson (225, 237). The chromatogram is treated in the following manner:

Reagent (*a*). 1. 0·5 c.c. acetylacetone in 50 c.c. butanol.
2. 5. cc. 50% aqueous potash in 20 c.c. alcohol.

0·5 c.c. of reagent 2 is added to 10 c.c. of reagent 1. The reagent is not stable, and must be freshly prepared each time.

Reagent (*b*). 1 g. *p*-dimethylaminobenzaldehyde is dissolved in 30 c.c. conc. HCl and treated with 180 c.c. butanol. Spray with (*a*), dry for 5 min. at 105°, then spray with (*b*), drying for 5 min. at 90°.

The test for free glucosamine is dark red, for *N* acetylglucosamine dark violet. Acetylhexosamines give the colour reaction without previous treatment with acetylacetone, and may thus be readily distinguished. Other sugars give only a weak reaction, which is hardly visible, with this reagent. Amino-acids are liable to cause serious interference with the reaction (165). Glucosamine also gives the ninhydrin reaction.

When estimating uronic acids it should be remembered that these are always present in equilbrium with their lactones. Equilibrium is constantly being established, and as a result of this, the more rapidly migrating lactone drags a tail of free uronic acid behind it, which thus smudges the chromatogram. The addition of ammonia to the phenol, however, displaces the equilibrium entirely to the side of the free acid, or rather its ammonium salt, and a well-defined, slowly migrating spot is then obtained which can be developed with silver nitrate. In acid solvents the uronic acids migrate as lactones and produce sharp spots. The test may, of course, be carried out just as well with aniline trichloracetate or other developers (see above).

Inosite may be rendered visible with silver nitrate–NaOH.

Glycosides are split into the free sugars under the conditions of the test reaction, and may then be identfied in one or other of the ways described above. They may also be identified by the amount of permanganate they consume (236). Bright spots on a violet background are formed on spraying with 1% $KMnO_4$ in 2% soda, subsequently heating. Oligosaccharides may

R_f-values of the Sugars (237)

	Phenol and 1% NH_3 HCN	Collidine	Butanol–acetic acid H_2O	Butanol 45% Ethanol 5% Water 49% Ammonia 1%	*n*-Butanol 1% NH_3	*iso*Butyric acid	Methyl ethyl ketone 1% NH_3
D-Glucose .	0·39	0·39	0·18	0·105	0·070	0·13	0·025
D-Galactose .	0·44	0·34	0·16	0·09	0·06	0·14	0·015
D-Mannose .	0·45	0·46	0·20	0·13	0·10	0·15	0·05
L-Sorbose .	0·42	0·40	0·20	0·12	0·085	0·16	0·05
D-Fructose .	0·51	0·42	0·23	0·135	0·10	0·18	0·045
D-Xylose .	0·44	0·50	0·28	0·17	0·125	0·19	0·09
D-Arabinose .	0·54	0·43	0·21	0·145	0·109	0·21	0·075
D-Ribose .	0·59	0·56	0·31	0·21	0·18	0·22	0·165
L-Rhamnose .	0·59	0·59	0·37	0·285	0·22	0·30	0·18
D-Desoxyribose	0·73	0·60	—	—	—	0·32	—
L-Fucose .	0·63	0·44	0·27	—	—	—	0·095
Lactose . .	0·38	0·24	0·09	0·00	0·00	0·07	0·00
Maltose . .	0·36	0·32	0·11	0·15	0·01	0·085	0·00
Cane-sugar .	0·39	0·40	0·14	—	—	—	—
Raffinose .	0·27	0·20	0·05	—	—	—	—
D-Galacturonic acid . .	0·13	0·14	0·14	—	—	—	—
D-Glucuronic acid	0·12	0·16 (0·72)	0·12 (0·32)	—	—	0·08 (0·22)	—
D-Glucosamin-hydrochloride	0·62	0·32	0·13 (0·17)	—	—	0·05 (0·20)	—
Chondrosamin-hydrochloride	0·65	0·28	0·12 (0·16)	—	—	—	—
N-Acetyl-glucosamine .	0·69	0·05	0·26	—	—	0·25	—
Ascorbic acid .	0·24	0·42	0·38	—	—	0·19	—
Dehydroascorbic acid . .	0·16	0·68	0·27	—	—	0·16	—
Inosite . .	0·23	0·10	0·09	—	—	—	—
Araboketose (322) . .	0·66	—	—	—	—	—	—
D-Lyxose (322) .	0·45	—	—	—	—	—	—
Xyloketose (322)	0·59	—	—	—	—	—	—
Rhamnoketose (322) . .	0·72	—	—	—	—	—	—
5-Methylthio-ribose (11) .	0·65	—	—	—	—	—	—
Sucrose	0·39	0·40	0·14.				

also be tested for in this manner. Iodine vapour constitutes a truly universal developer (205). Sugars, ascorbic acid, hydroxy- and keto-acids give white spots on a brown background, phenols and aromatic acids dark spots on a brown background. *cyclo*Hexa-amylose and *cyclo*hepta-amylose may be identified by their characteristic iodine reaction (78).

If it is desired to investigate the structure of an oligo or polysaccharide, the following procedure is employed: all free OH-groups are methylated, after which the compound is hydrolysed and the resulting methylated sugars are separated. The points at which the molecules were originally attached may then be inferred from the position of the free OH-groups. Separation

R_{TG}-*values of Sugars*

Solvent: *n*-butanol–ethanol–water (50 : 10 : 40 :), Whatman 1 paper (154).

Raffinose	0·001	4 : 6-Dimethylgalactose	0·42
Lactose	0·016	2-Desoxyribose, 2 : 6-dimethylgalactose	0·44
Maltose	0·021	4 : 6-Dimethylglucose	0·46
Cane-sugar	0·03	2-Methylfucose, 2 : 6-dimethylglucose	0·51
D-Gluco-1-galaoctose	0·038	3 : 4-Dimethylglucose, 4 : 6-dimethylaltrose	0·52
D-Gulo-1-galaheptose	0·050	2 : 3-Dimethylmannose	0·54
D-Gluco-*d*-guloheptose	0·053	2 : 3-Dimethylglucose, 4-methylrhamnose, 4 : 6-dimethylmannose	0·57
D-Manno-*d*-galaheptose	0·058	3 : 4-Dimethylmannose	0·58
D-Gala-1-glucoheptose	0·064	3-Methylchinovose	0·60
Turanose	0·060	3 : 4-Dimethylfructose, 2-desoxyrhamnose	0·61
Galactose	0·070	2 : 3-Dimethylarabinose, 2 : 3 : 4-trimethylgalactose	0·64
D-Gluco-1-talooctose	0·82	2 : 4-Dimethylxylose	0·66
Glucose	0·09	2 : 4 : 6-Trimethylgalactose	0·67
Sorbose	0·10	3-Methylaltromethylose	0·68
Mannose, mannoheptulose, D-gala-1-mannoheptose, D-gulo-1-taloheptose	0·11	2 : 3 : 6-Trimethylgalactose	0·71
Fructose, glucoheptulose, gulose, arabinose, tagatose	0·12	2 : 3-Dimethylxylose	0·74
Xylose	0·15	2 : 4 : 6-Trimethylglucose	0·76
4-Methylgalactose	0·16	3 : 4 : 6-Trimethylmannose	0·79
Altrose	0·17	2 : 3 : 6-Trimethylmannose	0·81
Idose, 6-methylgalactose	0·18	2 : 3 : 6-Trimethylglucose, 1 : 3 : 4-trimethylfructose	0·83
Talose, lyxose	0·19	3 : 4-Dimethylrhamnose	0·84
Ribose, fucose	0·21	2 : 3 : 4-Trimethylglucose	0·85
2-Methylglucose	0·22	3 : 4 : 6-Trimethylfructose	0·86
2-Methylgalactose	0·23	Cymarose	0·87
Riboketose, apiose, 2-desoxygalactose	0·25	Oleandrose, 2 : 3 : 4 : 6-tetramethylgalactose	0·88
3-Methylglucose, xyloketose	0·26	Tetramethyl-fructopyranose	0·90
6-Methylglucose	0·27	2 : 3 : 4-Trimethylxylose	0·94
Chinovose	0·28	2 : 3 : 5-Trimethylarabinose	0·95
Rhamnose, α-methylmannoside	0·30	2 : 3 : 4 : 6-Tetramethylmannose	0·96
3 : 4-Dimethylgalactose, 4-methylmannose, β-methylarabinoside	0·32	**2 : 3 : 4 : 6-Tetramethylglucose**	**1·00**
2-Desoxyallose	0·33	2 : 3 : 5 : 6-Tetramethylglucose, 2 : 3 : 4-Trimethylrhamnose, 1 : 3 : 4 : 6-Tetramethylfructose	1·01
2-Methyl-β-methylaltroside	0·34		
Rhamnoketose, 3 : 6-anhydroglucose, talomethylose	0·37		
2-Methylxylose, 2-methylarabinose	0·38		
2 : 4-Dimethylgalactose	0·41		

of methylated sugars by fractional crystallization is difficult, whereas it is readily achieved on the paper chromatogram. The actual chromatography is carried out in just the same way as for non-methylated sugars. The more methyl groups a sugar contains, the greater is its solubility in organic solvents and the greater its R_f-value. Tetramethylhexoses and trimethylrhamnose therefore possess the largest R_f-values. A difference in R_f-value of 0·03 suffices for separation and so, for example, 2 : 3 : 6-trimethylglucose and 2 : 4 : 6-trimethylglucose (difference 0·07) or 2 : 4-dimethylxylose and 2 : 3-dimethylxylose may still readily be separated.

The above table contains R_f-values of the free and methylated sugars with reference to tetramethylglucose (R_{TG}-values).

3. Quantitative estimation

(*a*) *According to the area of the spots as for the amino-acids.*—e.g. (126).

(*b*) *Photometric evaluation of spot area and intensity* (314a). Provid a colour reaction is carried out under exactly standardized conditions, it with the aid of calibration curves, possible to deduce the amount of sug from the intensity of the coloration. This may be done using the silv

FIG. 61.—Enzymatic hydrolysis of maltose with maltase. Course of reaction after definite intervals.

Solvent, butanol—pyridine—water, 6 : 4 : 3. Developed with silver nitrate (314a).

nitrate reaction and the photometer cited on p. 43 (314a). Fig. 61 shows chromatogram giving the course of enzymatic hydrolysis of maltose. Th chromatogram was developed with silver nitrate. A quantitative photo metric evaluation of this chromatogram is to be seen in Fig. 62.

1. Developing with silver nitrate–ammonia (314a).

A freshly prepared mixture of 0·2*N* silver nitrate and 5*N* ammonia (1 : 1) i sprayed on to the chromatogram from both sides. About 35 c.c. silver nitrat solution are required for spraying an area of 12 dm.[2] Whatman 1 paper. The spraye chromatogram is suspended in a drying-oven for 6 min. at 80°. During this time th paper scarcely turns brown, but the sugars already start to appear as light brow flecks. The reaction proper sets in on subsequently treating with a current of stean at 100°. The paper then turns a light brown and the spots dark brown. In thi state, however, the paper is not durable, since it rapidly darkens. In order to preven this, the chromatogram is treated for 10–15 min. with a freshly prepared solution o 10–15% sodium thiosulphate. The paper must afterwards be well rinsed under run ning water (4–5 hr.).

2. Photometric evaluation of the silver spots on the paper.

The chromatogram is cut into strips 4 cms. wide in the direction of running, these strips then being rendered transparent by immersion in a solution of bromonaphthalene in paraffin oil. The strips are placed between a pair of glass plates and illuminated from below by means of a small bulb. A photocell is now slowly drawn down each strip and the readings are plotted on a graph. The area of the resulting curves is then measured out with a planimeter. From a knowledge of the area and the calibration curve (see below), the concentration of the sugar may be calculated.

This method fails, however, if the quantities of sugar exceed 25γ. The formazane method may then be employed.

3. Plotting of calibration curves.

In order to obtain the most exact value possible, at least five different concentrations are applied to the chromatogram. The table below (p. 68) gives a survey of the mono-, di- and tri-saccharide concentration ranges suitable for plotting the graphs. The chromatogram is run for 24 hr. so that the sugars form the largest possible spots, thus allowing of a better reaction between the sugars and the spraying reagent. After developing, the spots are evaluated according to one or other of the methods described above. A mean is then taken of the five values obtained which, even after working with the utmost care, generally show variations of 3–5%.

(*c*) *Extraction followed by a micro-estimation.* The spots are first located on a parallel strip or with the aid of the wire brush, after which they are cut out and extracted. Estimation may then be carried out:

1. Using the Somogyis copper reagent (118, 287).

5 c.c. portions of the sugar solution being investigated, a control solution from the paper, a control solution from the flask and a known sugar solution of approximately the same concentration are introduced into four different flasks fitted with ground glass stoppers, each of which is then treated with 5 c.c. Somogyis's reagent (287). The mixtures are then heated for 25 min. in a boiling water-bath, after which they are immediately transferred for 10 min. to a bath at 35° and treated with 0·5 c.c. 2·5% KI solution

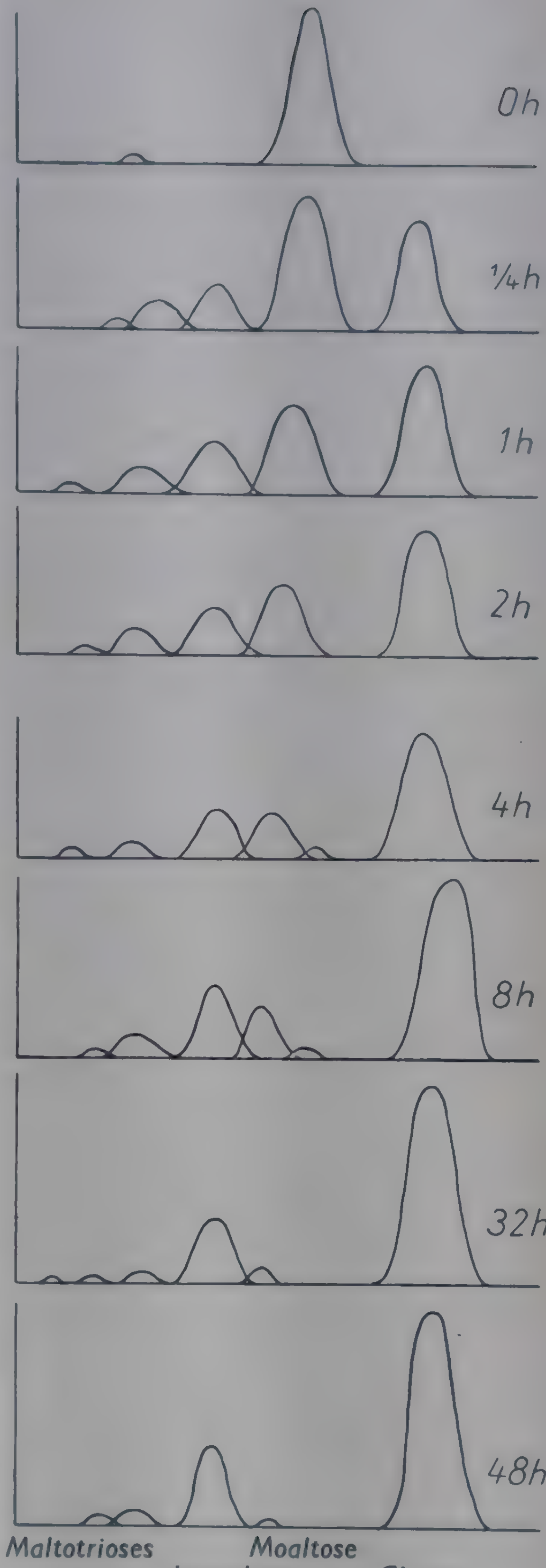


FIG. 62.—Quantitative photometric evaluation of the chromatogram in Fig. 61 (314a).

followed by 1·5 c.c. $3N$ H_2SO_4. The flasks are then cooled in cold water and titrat with $N/200$ thiosulphate solution. The quantity of sugar sought may now be ca culated from the standard and control values. An example of the application of th method may be found in (98).

Hagedorn and Jensen's method is too sensitive to impurities.

2. Periodate oxidation (153) with subsequent titration of the formic ac leads to fairly accurate results. An example illustrating the method therefore given (320):

0·1 c.c. of the extract to be investigated was applied to a starting line 1 cm. bro and 15 cm. long. A butanol–glacial acetic acid–water mixture, containing sufficie standard ferric acetate solution to ensure a total final concentration of 0·035 m Fe per 10 c.c. mixture served as solvent. After running for 50–60 hr., the paper w dried at 100° in a drying-oven, after which glucose and fructose became visible quite discrete light brown bands. They were cut out and extracted in a small Soxhl Each solution was then treated with 2 c.c. $0{\cdot}198N$ sodium metaperiodate, which react neutral to methyl red. The sodium periodate had been purified by recrystallizati from conc. nitric acid. After 24 hr. at 20°, 0·2 c.c. glycol was added to destroy exce of periodate and the formic acid formed was then titrated with $n/100$ NaOH in th presence of methyl red as indicator. The blank value for the paper was subtracte Standard values were calculated from the results obtained in experiments with know quantities of the sugar in question.

3. Titration using the Willstaetter–Schudel method (144) allows of a estimation of the sugars on the paper chromatogram with about 5% accurac

4. Colorimetric estimation of the formazane (313).

The solution is chromatographed on Whatman No. 1 paper, the solvent afterwar being well dried off. The paper is sprayed with a freshly prepared mixture of 2 aqueous triphenyltetrazonium chloride and $1N$ caustic soda, 1 : 1. The chromat gram is then developed for 20 min. in an atmosphere saturated with water vapour 40°, the excess TTC being subsequently washed out. The paper is then dried 25°. During all these operations, the background should, at the very most, turn light rose colour. The individual spots are cut out and extracted three times wi 5 c.c. portions of pyridine + 10% conc. HCl. For the subsequent photometr evaluation at 578 mμ, a calibration curve must be plotted and the blank value for piece of the paper of the same area subtracted.

The advantage of this process is that the colour is developed quantitatively on th paper itself. Thus the spot to be cut out may be seen from the very beginning.

Type of sugar	Elphor	TTC
Monosacch.	2–25 γ	5–100 γ
Di-Sacch.	5–50 γ	10–200 γ
Tri-Sacch.	10–100 γ	15–300 γ

4. Preparative application

The paper chromatographic separation of sugars on a preparative scale i carried out on cellulose columns (159). Cellulose powder is mixed to a pul with the solvent selected and the mixture is then poured into the tub Excess solvent is allowed to drain off, after which the solution is run in an

chromatographed. A fraction cutter separates the fractions trickling out, their individual composition then being tested on separate paper chromatograms. Methylated sugars may also be worked up in this manner (160).

In order to test the uniformity of the filling in the column, a suitable dyestuff dissolved in the same solvent may be run through, when this should pass down as a clearly demarcated band. So as to be able to follow the progress of the zones in the chromatogram proper, a dyestuff having a somewhat larger R_f-value than that of the fastest migrating sugar present is added. A previous run on a one-dimensional chromatogram is necessary to establish this. The time of emergence of the substance sought may thus be approximately judged. In addition to the solvents normally employed, the following mixtures are especially suitable: butanol–water, butanol–acetic acid, aqueous *iso*propyl alcohol, aqueous acetone and aqueous ethanol. Sugars which are found to run too quickly in these solvents, e.g. the tetramethylhexoses and the methylated pentoses, may be run in petrol ether (b.p. 100–120°)–butanol 60 : 40.

5. A selection of results

The occurrence of free sugars in numerous natural products has been investigated (38, 186, 237, 320) as well as the sugar content of urine, insects' blood, etc. (102, 195). The constituents of plant and animal polysaccharides may be ascertained relatively simply on the paper chromatogram. D-Tagatose, D-galactose and L-rhamnose have been isolated from *Sterculia setigera* (152). The substance specific to the blood groups contains fucose, galactose and *N* acetylglucosamine (5, 41, 237). The paper chromatographic study of specific polysaccharides should yield a number of interesting results, since the method represents an essential improvement in the detection of minute quantities of rare sugars (317). The quantitative elucidaton of many plant polysaccharides has already been carried out, e.g. (17, 155).

N-glycosides and the condensation products of sugars with amino-acids may also be investigated on the paper chromatogram (240).

If chromatography is carried out at 37°, a still sharper separation than that achieved at room temperature is possible, since the spots run at a higher concentration, and consequently remain smaller.

Paper chromatography has also played a decisive part in studies relating to CO_2 assimilation. The green alga Chlorella is submitted to radio-active $^{14}CO_2$, the compounds formed after definite intervals then being run on chromatograms and investigated radiographically. After 30 sec. most of the radioactivity was found in phosphoglyceric acid, triose phosphate and hexose phosphate. Cane-sugar was the most active compound after 90 sec., this being the first free sugar to be formed and arising from fructose-6-phosphate and glucose-1-phosphate (63, 64). After this, sugar synthesis and degradation take place according to the scheme in (18).

The two-dimensional method with phenol and butanol–acetic or pro ionic acid is employed here. Autoradiography is carried out according the method described on p. 32.

6. Paper electrophoresis of sugars (72, 166)

As is well known, certain sugars form esters with boric acid, provid these possess *cis*hydroxy groups. By the addition of a borate to the solve used to run the chromatogram, it is thus possible to influence preferential the speed of migration of individual sugars (177, 6).

In the presence of a borate buffer, sugars migrate to the anode in electrical field.

In buffers containing no borate, no migration takes place. Sugars may separated provided their mobility differs by 20% or more. Glucose ar galactose, which are difficult to part in a normal chromatogram, may readi be separated at pH 6 with a buffer. In the presence of a borate at the san pH, hexosamines behave just like neutral molecules, since they then car both an acidic and a basic function. Thus they do not migrate. Gl curonic acid migrates 1·4 times as fast as glucose.

This method is particularly useful for the investigation of proteins co taining polysaccharides. The borate electrophoresis may be utilized separate small quantities of sugars from large quantities of amino-acids ar peptides. At pH 8·6 with a borate buffer, only the acidic peptides, i.e. tho containing glutamic and aspartic acids, migrate at more or less the same spe as the sugars. The acidic amino-acids migrate more rapidly than the sugar This method is frequently suitable for the investigation of substances co taining only a few per cent of polysaccharide.

*Mobility of the Sugars (cm.^{2}/V. sec. × 10^5) in Borate Buffer, 0·2*M *Bor acid or 0·05 Borax respectively. Whatman 1* (72)

pH	7·0	8·0	8·6	9·2	9·7
Fructose	8·2	9·7	11·4	12·5	13·1
Sorbose	8·7	10·4	12·2	14·1	14·3
Glucose	2·4	6·5	11·4	14·5	14·6
Galactose . . .	2·8	5·8	9·6	13·0	13·1
Mannose . . .	2·6	4·9	7·8	9·8	10·0
Ribose	7·0	9·1	10·2	10·9	11·0
Arabinose . . .	3·2	6·5	10·3	13·3	13·9
Rhamnose . . .	1·3	2·4	4·4	7·1	7·8
Cellobiose . . .	0·5	0·5	1·5	3·2	4·5
Raffinose . . .	0·5	0·9	1·7	3·6	4·8

Alcohols, Sugar Alcohols

The sugar alcohols reduce ammoniacal silver nitrate solution on warming, the reaction being especially sensitive in the presence of NaOH, so that it is possible to detect as little as 1γ by this method (161).

The following processes, which are suitable for developing all 1 : 2-glycols, are elegant and of general application (51).

1. Periodate oxidation, followed by detection of the resulting aldehyde with fuchsine–sulphurous acid.

Solution A: 2% aqueous metaperiodate; Solution B: 1 g. rosaniline in 50 c.c. water, decolorized with SO_2 and made up to 1 lit. The chromatogram is dried for 5 min. at 80°, sprayed with solution A and maintained for 7 min. at 60° in an atmosphere of nitrogen. It is then treated with SO_2 until the iodine liberated has been consumed and subsequently sprayed with solution B. Red spots become visible after 3 hr., their appearance being hastened by heating to 60°. Nucleosides are treated with 2% periodiate in air for 10 min. at 20° and then left a further 10 min. at 90° with B.

2. Periodate oxidation. Formic acid may then be detected with glucose, glycerol, mannitol, sorbitol, cane-sugar and maltose.

Solution A: as above; C: 10% aqueous ethylene glycol; D: 5% aqueous potassium iodide. The periodate oxidation is carried out with A as described above. C is then sprayed on and the paper is brought for 10 min. into an atmosphere of nitrogen, which destroys excess periodate. On spraying the paper with D, iodine is liberated at the acidic patches, as a result of the reaction between the iodide and iodate in the presence of the formic acid formed from the 1 : 2 : 3-trioxy-compounds. In this way, brown spots are produced against a white background.

3. Lead tetra-acetate oxidation.

1 g. lead tetra-acetate in 100 c.c. benzene, shake with animal charcoal, filter. The dry paper is moistened with a little xyline, sprayed with the reagent and dried at room temperature. Wherever glycols are present, the lead reverts to the bivalent state, brown PbO_2 being precipitated on the rest of the paper. White patches on a brown background.

The table below gives a list of the compounds which have been identified in this manner, together with their respective R_f-values.

Lower-alcohols are converted into the 3 : 5-dinitrobenzoates, the latter then being chromatographed (215): The paper is steeped in a solution of rhodamine (20 mg./l.) and dried at 100°. The mixture is then applied to the paper, which is suspended overnight in a cabinet saturated with methanol and hexane vapour. The run is not commenced until the following morning, using methanol saturated with hexane as solvent. After drying, dark spots on a yellow fluorescent background.

R_f-values of Dinitrobenzoates at 20°. Whatman 1

Methanol	0·24	*iso*Butanol	0·61
Ethanol	0·39	2-Methylpropanol-1	0·55
Propanol	0·46	Pentanol-1	0·66
*iso*Propanol	0·51	3-Methylbutanol-1	0·65
Butanol	0·57	Hexanol-1	0·72

R_f-*values of Sugar Alcohols* (*161*)

	Butanol–H_2O	Butanol–ethanol–water (4 : 1·1 : 1·9)	Benzol–*n*-butanol–pyridine–water (1 : 5 : 3 : 3)	Butanol–acetic acid–water (5 : 1 : 2)	Butanol–ethanol–water (4 : 1 : 5)
Trimethylenglycol	0·67	0·62	0·63	—	0·63
Glycol	0·51	0·54	0·58	0·58	0·61
Glycerol	0·30	0·43	0·46	0·44	0·37
α-Methylgalactoside	0·13	0·31	0·35	0·31	0·18
α-Methylmannoside	—	—	—	—	0·31
β-Methylmannoside	—	—	—	—	0·03
Sorbitol	0·06	0·21	0·21	0·17	0·10
Dulcitol	0·05	0·21	0·20	0·18	0·10
Mannitol	0·05	0·22	0·22	0·19	0·10
Inositol	0·00	0·10	0·07	0·05	0·02
Cane-sugar	0·00	0·15	0·18	0·09	0·03

R_f-*values of Glycols* (*on Whatman No. 1*) (*51*)

	Butanol–acetic acid–water (4 : 1 : 5)	Butanol–acetic acid–water–conc. HC (20 : 5 : 25 : 1)
Glucose	0·19	—
Glycol	0·64	0·69
Glycerol	0·48	0·52
Mannitol	0·21	0·26
Sorbitol	0·19	0·25
Inositol	0·09	—
Lactose	0·04	—
Maltose	0·05	—
Raffinose	0·03	—
Cane-sugar	0·08	—
Trehalose	—	0·03 *
K-Glucose-1-phosphate	0·03	—
α-Methylglucopyranoside	0·34	—
Gluconic acid	0–0·24	~0·18
DL-Tartaric acid	—	0·48
Mesotartic acid	—	0·41
Erythro-9 : 10-dihydroxystearic acid	—	0·52–0·94
Erythro-6 : 7-dihydroxystearic acid	—	0·91
Citric acid	—	0·45 *
Lactic acid	—	0·68–0·78 *
Pyruvic acid	—	0·77 *
Dihydroxyacetone	—	0·38 *

* On Whatman No. 54.

Reductone and Related Compounds

These may be detected on the paper chromatogram by the fact that they reduce a neutral solution of silver nitrate in the cold. They may also be rendered visible with Tillmann's reagent (2 : 6-dichlorophenol-indophenol) (239, 323). It is apparent that reductone is formed not only from glucose and xylose but also from fructose, arabinose and rhamnose. Presumably no reductone formation takes place in the bacteria themselves.

The titanium reaction may also be used to demonstrate the presence of endiols on the paper chromatogram (324). For this purpose, the dry paper is first lightly sprayed with a mixture of 50 vols. methanol and 50 vols. pyridine, followed by a 5% $TiCl_3$ solution in water. On suspending in the air, the initial brown-black coloration gradually disappears, its place being taken by the stable orange coloured spots of the endiol compounds on a colourless background.

R_f-values of Reductones and Related Compounds (239, 323)

	Butanol–acetic acid–water (4 : 1 : 5)	Butanol–acetic acid–water (10 : 2 : 5)	1% Acetic acid saturated with aqueous phenol	Trace KCN collidine
Ascorbic acid	0·37	0·31–0·36	0·35	0·40
*iso*Ascorbic acid	0·30	—	0·40	0·41
Hydroxytetronic acid	0·63	—	0·62	0·49
Reductone	0·63	0·61	0·66	0·46
Dehydroascrobic acid	0·41	—	0·38	0·44
Dihydroxyacetone	—	0·41	—	—

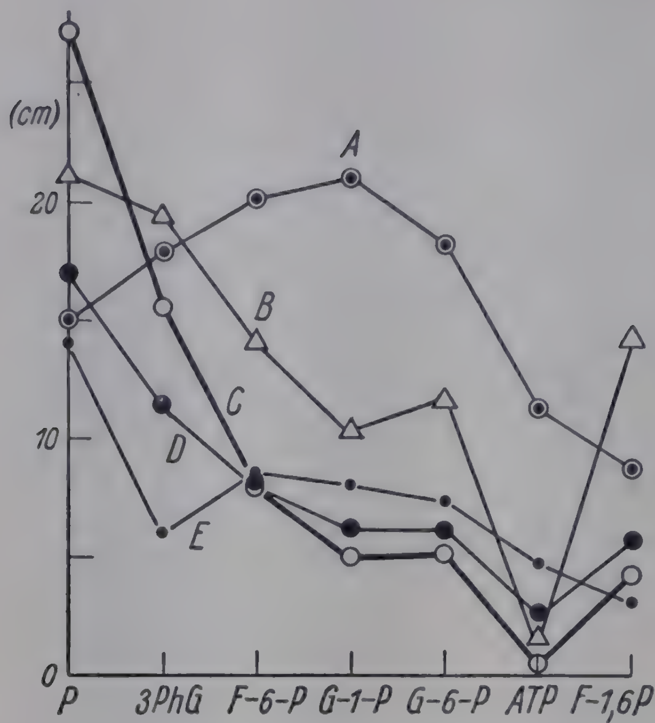
Phosphoric Acid Esters (139)

Since the organic phosphoric acid esters are generally very soluble in water, they migrate very slowly on the paper chromatogram. Continuous running chromatograms are therefore usually employed.

The completed chromatogram is dried at 70° and developed as follows:

Spraying: The solution is prepared as follows: 5 c.c. 60% perchloric acid, 10 c.c. 1*N* hydrochloric acid and 25 c.c. 4% ammonium molybdate solution are mixed and made up to 100 c.c. with water. Approximately 1 c.c. of this solution is employed per sq. dm. paper. The sprayed chromatogram is dried in warm air, and subsequently maintained at 85° for 7 min. On treating with H_2S, spots of molybdenum-blue now make their appearance.

For the quantitative determination of phosphoric acid esters, the paper must be washed out in succession with 2*N* acetic acid, distilled water and



P = free orthophosphate
3 PhG = 3-phosphoglyceric acid
F-6-P = fructose-6-phosphate
G-1-P = glucose-1-phosphate
G-6-P = glucose-6-phosphate
ATP = adenosine triphosphate
F-1, 6-P = fructose-1 : 6-phosphate
A in propanol–ammonia–water 6 : conc. : 1 ; 15h. run
B in *tert.*-butanol–water–picric ac 80 : 20 : 4 g ; 15h. run
C in *iso*propyl ether–90% formic aci 9 : 6 ; 6h. run
D in *tert.*-amyl alcohol–water–form acid 9 : 9 : 9 ; 15h. run
E in ethyl acetate–pyridine–water 100 45 : 100 ; 23h. run

FIG. 63.—Speeds of migration of various phospho-sugars (139).

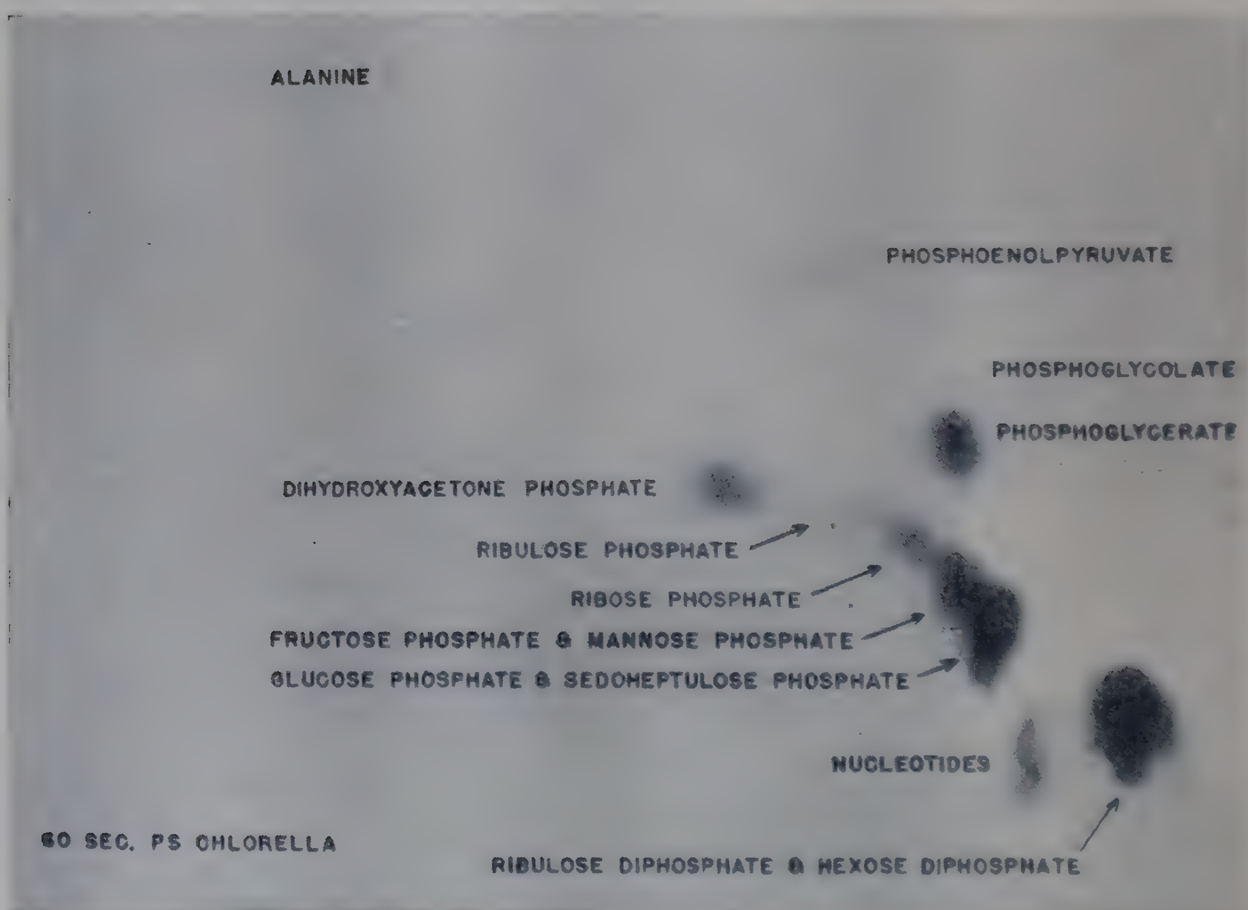


FIG. 64.—Two-dimensional chromatogram of hexose- etc. phosphates with ^{32}P. Kindly placed at our disposal by Dr. A. A. Benson.

8-hydroxyquinoline. This treatment ensures the removal of all traces of phosphate.

Since it is not possible to give R_f-values for a continuous running chromatogram, a survey of the speed of migration of various sugar phosphates is appended (139).

Fructose phosphate may also be developed with naphtho-resorcinol (312). Radio-actively labelled phosphates in CO_2 assimilation products may be separated on a two-dimensional chromatogram with phenol and phenol–acetic acid.

Purines, Nucleosides, Nucleotides, Nucleic Acids, Pterines, Flavins

1. Developing

Nearly all these substances can be recognized by their characteristic fluorescence or absorption in uv-light. The strip is either held directly under the uv-lamp or photographed in uv-light (206, 207). Purines and pyrimidines form mercury salts when treated with mercuric chloride in dilute solution. Excess sublimate is subsequently removed by washing, the paper then being treated with H_2S. This precipitates HgS in black patches. Radio-active nucleotides may be detected radiographically (164, 261). Adenylic, guanylic and cytidylic acids may be detected with uranyl salt and ferrous cyanide (308). Nucleosides and nucleotides possessing glycol groups can be developed by the periodate method, as already described (v. p. 71). The photoprint process (206, 207) is, however, the most convenient method. All purines, including those which do not fluoresce, absorb uv-light in the region of 2600 Å. Dark patches, which, of course, give rise to white ones on the photographic print, therefore make their appearance in passing filtered uv-light through the paper. Uv-light of about

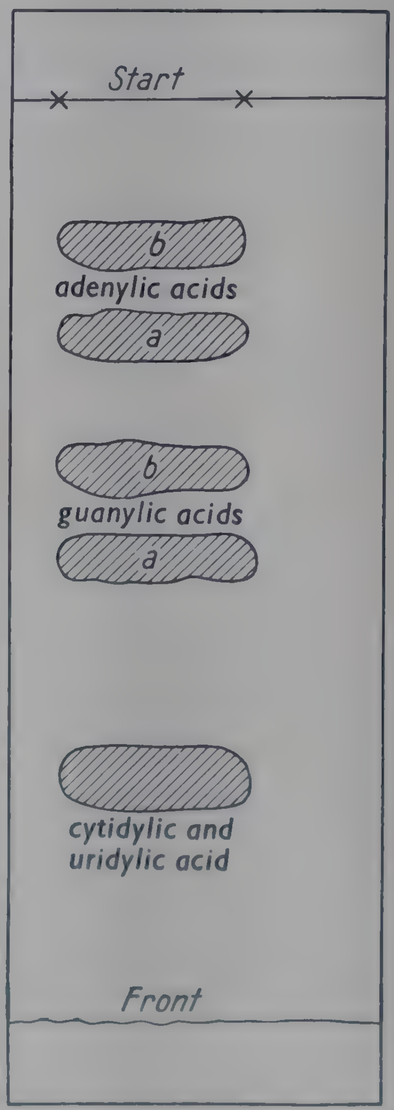


Fig. 65.—Chromatogram of the product resulting from alkaline hydrolysis of ribonucleic acid (208).

Solvent: saturated ammonium sulphate solution 79%, water 19%, isopropanol 2%.

2600 Å. may be filtered out from a mercury-vapour lamp in the following manner:

1. A quantity of solution of composition 350 g. $NiSO_4$, $7H_2O$ and 100 g. $CoSO_4$, $7H_2O$ in 1000 c.c. water is filled into a 3-cm.-thick glass filter container of diam. 2·2 cm. fitted with quartz cover-glasses.
2. A 3·5-cm.-long glass filter container containing a little $CaCl_2$ is filled with chlorine gas.

R_f-*values of the Nucleosides*

Substance	Solvent	R_f	Author
Flavin-adenine-dinucleotide	Butanol–acetic acid	0·02	(79)
,, ,,	Collidine	0·11	
Riboflavin-phosphate	Butanol–acetic acid	0·09	
,, ,,	Collidine	0·09	
Riboflavin	Butanol–acetic acid	0·30	
,,	Collidine	0·50	
Thymine	Butanol–NH_3	0·54	(158)
Thymidine	,,	0·51	
Uracil	,,	0·35	
Cytosine	,,	0·26	
Cytidine	,,	0·16	
Adenine	,,	0·45	
Adenine-desoxyriboside	,,	0·33	
Guanosine	,,	0·10	
Guanine-desoxyriboside	,,	0·21	
Xanthine	,,	0·01	
Hypoxanthine	,,	0·18	
Thymine	Butanol–water	0·47	(311)
Uracil	,,	0·32	
Cytosine	,,	0·19	
Adenine	,,	0·28	
Guanine	,,	0·07	
Xanthine	,,	0·07	
Hypoxanthine	,,	0·17	
Thymidine	,,	0·45	(182)
Cytosine-desoxyriboside	,,	0·21	
Adenine-desoxyriboside	,,	0·32	
Guanine-desoxyriboside	,,	0·18	
Hypoxanthine-desoxyriboside	,,	0·21	
2-Amino-6-hydroxypteridine-8-carboxylic acid	*sec*.-Butanol–90·0 formic acid–water 8 : 2 : 5	0·40	(321)
2-Amino-6-hydroxypteridine-9-carboxylic acid	,, ,,	0·29	
Hypoxanthine-thiomethylpentoside	Butanol–water	0·40	(11)
Adenine-thiomethyl-pentoside	,,	0·58	
2-Amino-3-hydroxypyridine	Petrol ether (90–100°)–butanol–water 1 : 2 : 3	0·68	(42)
2-Amino-6-hydroxypyridine	,, ,,	0·63	

R_f-*values of the Purines and Pyrimidines* (206)

	Butanol 84 Water 14	Butanol 84 Water 14 + 5% NH_3	Butanol 77 Water 13 Formic acid	Butanol 50 Water 35 Ethanol 15	Amyl alcohol saturated with H_2O	Amyl alcohol saturated with H_2O + 5% NH_3	Amyl alcohol saturated with H_2O 90, formic acid 10
Hypoxanthine	0·26	0·12	0·30	—	0·15	0·02	0·19
Xanthine	0·18	0·05	0·24	—	0·10	0·00	0·16
6 : 8-Dihydroxypurine	0·18	0·04	0·24	—	0·07	0·00	0·11
Uric acid	0·01	0·00	0·14	—	0·00	0·00	0·06
3-Methylxanthine	0·29	0·13	0·32	—	0·19	—	—
Theophylline	0·52	0·22	0·64	—	0·54	0·12	0·55
Theobromine	0·42	0·27	0·47	—	0·28	0·15	0·36
3 : 8-Dimethylxanthine	0·42	0·18	0·48	—	0·34	—	—
Coffein	0·63	0·65	0·71	—	0·56	0·50	0·67
Adenine	0·38	0·28	0·33	0·55	0·28	0·16	0·12
Guanine	0·15	0·11	0·13	0·37	0·05	—	0·04
Adenosine	0·20	0·22	0·12	0·50	0·11	0·09	0·04
Guanosine	0·15	0·03	0·17	0·40	0·02	0·00	0·04
Cytosine	0·22	0·24	0·26	0·53	0·09	0·09	0·07
Uracil	0·31	0·19	0·39	0·46	0·22	0·08	0·23
Thymine	0·52	0·35	0·56	—	0·40	—	—
Cytidine	0·12	0·11	0·18	0·42	0·02	0·03	0·03
Uridine	0·17	0·08	0·25	0·49	0·05	0·03	0·07

R_f-*values of Nucleosides* (51)

	R_f in *n*-Butanol–water	R_f in Collidine–water
Adenosine	0·14	0·68
Cytidine	0·13	0·50
Guanosine	0·09	—
Uridine	0·21	0·81
Guanine-desoxyriboside	0·17	0·66
Hypoxanthine-desoxyriboside	0·19	—
9-α-*d*-Arabofuranosyl-adenine	0·14	—
9-β-*d*-Glucopyranosyl-adenine	0·08	0·36
9-β-*d*-Mannopyranosyl-adenine	0·09	0·42
9-β-*d*-Riboglucopyranosyl-adenine	0·12	0·42
2′-Desoxy-*d*-glucopyranosyl-theophylline	0·30	—
2′-Desoxy-*d*-ribopyranosyl-theophylline	0·40	—
2′-Desoxy-*d*-gluco-pyranosyl-uracil	0·22	—
1-*d*-Arabopyranosyl-benzimidazol	0·62	0·78
1-*d*-Glucopyranosyl-benzimidazol	0·49	0·82
2′ : 3′-*iso*Propyliden-adenosine	0·59	—

These filters only allow the passage of the mercury lines of 2537 and 2650 Å.

A piece of photographic printing paper is placed on a convex wooden block and the dried chromatogram is pinned down on top of it with drawing-pins. The time of exposure and distance from the source of illumination

will depend entirely on the strength of the lamp and sensitivity of th paper. The great advantage of this method is that the substances remai unchanged and may thus be employed for a subsequent quantitativ estimation.

The extinction of fluorescence by uv-absorbing substances may also b utilized in a similar manner (337). The paper is sprayed with a 0·005% solution of fluorescein in 0·5% ammonia and then observed under uv-light filtered as above, without completely drying. The paper then exhibits greenish-yellow fluorescence, while the spots appear as dark areas. Th lower limit of detection lies between 5 and 10γ.

2. Solvents

The solvents are listed in the tables of R_f-values. Nucleotides—e.g adenosine phosphate—scarcely migrate at all in organic solvents on accoun of their great solubility in water. It is therefore convenient to work with aqueous buffer solutions in a two-phase mixture (65a).

Although the nucleotides migrate with buffer solutions alone, it is no possible to obtain well-defined spots in a single phase. If, however, a film of organic solvent covers the buffer solution, the spots remain clearly defined.

Solution A: 5% aqueous *sec.* Na phosphate.
Solution B: *iso*amyl alcohol.

For running ascending chromatograms, the bottom of a sufficiently large vessel is covered by a 1 cm. layer of the aqueous buffer, on to which is floated a 0·5-cm. layer of the organic solvent. The chromatogram is then suspended with its lower end dipping into this two-phase mixture. The R_f-values are found to vary somewhat with the depth of the organic layer and also with the depth to which the paper is immersed in the buffer solution (= 1 cm.); these factors should therefore be maintained as constant as is practicable.

This method may be employed to separate the pyrimidine nucleotides on the paper chromatogram.

R$_f$-*values of Nucleotides and Nucleic Acids by the Two-phase Process (65a, 285a) with* sec. *Na Phosphate and* iso*Amyl Alcohol*

Cytidylic acid	0·86	Adenylic acid b	0·64
Uridylic acid	0·86	Adenosine triphosphate (ATP)	0·82
Guanylic acid	0·79	Adenosine diphosphate (ADP)	0·80
Adenylic acid a	0·74		

On employing *n*-amyl alcohol containing 0·5% laurylamine instead of *iso*amyl alcohol, the definition of the spots is enhanced (285a).

Nucleotides with n-*Amyl Alcohol Laurylamine and* sec. *Na Phosphate* (285*a*)

Inosyl acid	0·86	Adenosine	0·48
Adenosine-(2)-phosphate	0·69	ADP	0·76
Adenosine-(5)-phosphate	0·69	ATP	0·83
Adenosine-(3)-phosphate	0·61		

When working two-dimensionally, butanol saturated with 10% aqueous urea is used for running in the second direction.

3. A selection of results

The nucleic acids in yeast and in the pancreas (310), in tubercles (309) and in various other animal and plant tissues have been investigated (138). A separation of yeast–ribonucleic acid and muscle–adenylic acid has been achieved on the paper chromatogram (308). The course of the action of crystallized desoxyribonuclease on calf's thymus and yeast has been followed and the individual products of cleavage isolated (360). The transference of radio-active inorganic phosphate into the nucleotides has been investigated on liver homogenate (164). As a result of these investigations, it was established that the phosphate in aerobic phosphorylation is principally bound as flavin–adenine–dinucleotide and secondarily as adenosine triphosphate, while the flavin mononucleotides, the pyridine and adenylic acid exhibit no radioactivity. Purine labelled with ^{15}N may be employed in a similar manner to study the purine transformations in the organism (261). It is advantageous to test the purity of substances prepared synthetically on the paper chromatogram (281, 321). A method for the paper chromatography of the pterines has also been developed (128, 150).

4. Preparative application

The chromatography of purine derivatives has been carried out on starch columns of length 20 cm. and diam. 2·3 cm. A number of solvents proved suitable (82, 103, 260).

5. Paper electrophoresis

of the nucleotides (306, 337, 94) is carried out with an acid acetate buffer at about p_H 3·2 in one of the electrophoresis chambers described. Uridylic acid migrates most rapidly, followed by guanylic, adenylic and cytidylic acids. In this buffer, nucleo-bases and nucleosides migrate to the cathode. Adenine migrates most quickly in this instance, followed by guanine, urocil and cystosine. The sequence for the nucleosides is cytidine, adenosine, uridine and guanosine. The two last-named scarcely separate. By means of a pre-separation with a borate buffer at p_H 9, in which the nucleosides and nucleotides migrate to the anode, a separation into three groups may be achieved.

Individual treatment of these then leads to complete separation. In a bora buffer at p_H 9·2, the nucleotides naturally migrate most rapidly, followed b the nucleosides, whereas the nucleo-bases fail to migrate at all.

6. Quantitative estimation

On account of the strong uv-absorption of all the purine derivatives, th spectral–photometric estimation is the easiest and most suitable metho (208). The spots, the position of which has been ascertained by photograph in uv-light, are cut out and extracted for 18 hr. with $n/10$ hydrochloric acid The composition of the solutions is then determined in a photometer, e.g Beckman's.

Molar Extinction Coefficients (208)

Compound	Wave length, mμ	$\epsilon \times 10^{-3}$
Guanine	250	11·0
Adenine	260	13·0
Cytidylic acid	280	12·3
Uridylic acid	260	9·45

Phenols

1. Developing

Phenolic compounds may, assuming they possess no colour of their own be detected by their uv-absorption, the ferric chloride reaction, or their ability to couple (diazotized sulphanilic acid). The reactions are generally so characteristic that it is a comparatively simple matter to prove the presence of phenols on the paper chromatogram. Apart from differing R_f-values there are also a number of other possibilities for distinguishing the majority of the individual phenols.

Diazotized sulphanilic acid: 50 g. sulphanilic acid are dissolved in 250 c.c. 10% caustic potash, cooled and then treated with 200 c.c. 10% sodium nitrite solution. This mixture is then run out from a dropping funne into ice-cooled hydrochloric acid (80 c.c. conc. HCl + 40 c.c. water). The precipitated diazonium salt is filtered at the pump, washed with ice-water alcohol and ether and cautiously dried in the air. Directly before spraying a solution of 0·1 g. diazonium salt in 20 c.c. 10% soda solution is prepared It may also be found necessary to make up this solution in 50% ethanol.

Ferric chloride reaction: 2% solution. Green, blue or brown spots.

Saturated ammonium vanadate solution: This brings up olive-green to black spots. The spots change their colour in a characteristic manner on spraying with 1N sulphuric acid.

Silver nitrate solution is reduced by polyphenols.

	Solvent	R_f-value in solvent indicated on left	R_f-value in butanol sat. with conc. aq. bicarbonate soln. on paper buffered to p_H 8 (99)	Author
Vanillin	Petrol ether (100°) sat. with water	0·32	—	(26)
Syringaaldehyde	,, ,,	0·12	—	(26)
,,	Butanol-50–pyridine-50, sat. NaCl soln.	1·00	—	(26)
Phenol	,, ,,	0·97	0·95	(110)
p-Hydroxybenzoic acid	,, ,,	0·87	0·25	(110)
m-Hydroxybenzoic acid	,, ,,	0·74	0·21	(110)
Salicylic acid	,, ,,	0·65	—	(110)
Catechol	,, ,,	0·96	0·91	(110)
Protocatechuic acid	,, ,,	0·74	—	(110)
Resorcinol	,, ,,	0·97	—	(110)
β-Resorcylic acid	,, ,,	0·50	—	(110)
Hydroquinone	,, ,,	0·96	—	(110)
Gentisic acid	,, ,,	0·51	—	(110)
Pyrogallol	,, ,,	0·94	0·19	(110)
Pyrogallol carboxylic acid	,, ,,	0·71	—	(110)
Gallic acid	,, ,,	0·43	0·05	(110)
Phloroglucinol	,, ,,	0·96	—	(110)

R_f-*values of the Phenols* (*14*)

	R_f in butanol–acetic acid	R_f in cresol	Ferric chloride reaction	Silver nitrate reaction
Benzoic acid	0·92	0·93	—	—
Catechol	0·91	0·74	black	++
Cinnamic acid	0·94	0·92	yellow	—
Coumaric acid	0·94	0·82	orange	+
Gallic acid	0·69	0·08	grey	++
m-Hydroxybenzoic acid	0·91	0·72	pale yellow	—
p-Hydroxybenzoic acid	0·90	0·72	yellow	—
Orcinol	0·91	0·75	grey	+
Phloroglucinol	0·76	0·16	grey	+
Phloroglucinol carboxylic acid	0·55	0·06	grey	—
Pyrocatechuic acid	0·85	0·35	pale yellow	++
Pyrogallol	0·77	0·38	reddish-brown	++
Hydroquinone	0·88	0·69	grey	+
Resorcinol	0·91	0·63	grey	+
β-Resorcylic acid	0·93	0·54	purple	—
Salicylic acid	0·95	0·81	purple	—
Vanillic acid	0·92	0·81	yellowish-brown	—

Anthocyanidins etc. (14)	R_f in butanol–acetic acid	R_f in cresol
Monohydroxybenzal-acetophenones	1·00	—
Chrysin	0·97	1·00
Dihydroxybenzal-acetophenones	0·97	—
Galangin	0·92	0·92
Apigenin	0·92	—
Kaempferol	0·91	0·62
Cyanomaclurin	0·90	—
Luteolin	0·89	0·68
Morin	0·88	0·63
Butein	0·85	0·63
Apigenidin	0·82	1·00
Pelargonidin	0·80	—
D-Catechin	0·76	—
Quercetin	0·73	0·26
Fisetin	0·71	0·40
Cyanidin	0·68	—
Gallocatechin	0·46	—
Myricetin	0·42	0·06
Quercagetin	0·39	0·08
Delphinidin	0·37	—
Diosmetin	0·88	—
Hirsutidin	0·73	—
Paeonidin	0·73	—
Malvidin	0·54	—

Anthocyanins (14)	R_f in butanol–acetic acid
Delphinidin-3-monoglucoside	0·16
3 : 5-Diglucoside	0·10
Malvidin-3-monoglucoside	0·40
3 : 5-Diglucoside	0·22
Cyanidin-3-monoglucoside	0·15
3 : 5-Diglucoside	0·37
Paeonin-3-monoglucoside	0·26
3 : 5-Diglucoside	0·46
Hirtsutidin-3-monoglucoside	0·48
3 : 5-Diglucoside	0·62
Pelargonidin-3-monoglucoside	0·37
3 : 5-Diglucoside	0·60
*iso*Quercitrin	0·68
Quercimeritrin	0·59
Phloridzin	0·80
Phloretin	0·97
Iridin	0·73
Tannins:	
D-Catechin	0·76
DL-Catechin	0·74
L-Epicatechin	0·65
DL-Gallocatechin	0·57
L-Gallocatechin	0·47
L-Epicatechin-gallate	0·86
L-Gallocatechin-gallate	0·72

Fructose solution yields coloured spots on warming with phenols (see section on phenols).

Phosphomolybdic acid (263) permits of differentiation between *o*- and *m*-diphenols.

Anthocyanins give a violet to blue reaction with ammonia, anthoxanthins an intense yellow to orange coloration.

Millon's Reagent (99): 1 part (wt.) mercury is dissolved in 2 parts fuming nitric acid. The solution is diluted with 2 vols. water. The chromatogram is given an initial light spraying, dried at 35° and then re-sprayed. After about 5 hr. the yellow to brown spots will have reached their full intensity.

2. Solvents

Only acidic solvents are of any use here, chiefly butanol–acetic acid–water. The following mixture is also very useful (278): 50 c.c. water, 40 c.c. butanol and 10 c.c. glacial acetic acid are mixed and the organic phase is separated off. 50 c.c. of this phase is then treated with 5 c.c. glycol. Further solvents are: *m*-cresol–acetic acid–water 50 : 2 : 8 (14) benzene–glacial acetic acid–water (40), and others.

The various tannins are best separated with water saturated *sec*-butanol (178).

R_f-values of the Tannins (278)

Solvent: butanol–acetic acid–glycol (see above).

	R_f values	Ferric chloride reaction	Diaz. sulphanilic acid. Initially all yellow + NH_3	Vanadate. Initially all olive-brown + H_2SO_4
Chebulic acid	0·64	blue	colourless	reddish-violet
Chebulagic acid	0·43	,,	,,	blue
Sumach	0·78	,,	,,	colourless
Gallic acid	0·79	,,	dark brown	blue-brown
'Spaltsäure'	0·41	,,	colourless	blue, colourless
Hamamelitannin	0·57	,,	,,	colourless
3 : 6-Digalloylglucose	0·50	,,	,,	blue, colourless
3-Galloylglucose	0·47	,,	,,	,,
6-Galloylglucose	0·47	,,	,,	,,
Glucogallic acid	0·53	,,	,,	,,
Glucose	0·41	,,	—	—

Phenols which are found to run too quickly in the solvents instanced—e.g. naphthols—may be chromatographed using aqueous carbonic acid (12a).

Carbon dioxide is bubbled through distilled water at 21° until a p_H of 4·2 has been reached. The chromatogram should then be run at approximately the same temperature.

3. Results

The flower pigments may readily be isolated from various plants, these then being estimated colorimetrically (13, 125). Paper chromatographic control during the synthesis of anthocyanins is also of great value (266). The degradation products of lignin may be isolated (12, 13, 26, 123, 292). Paper chromatography has proved especially valuable for the separation of tannins (37, 178, 278, 325).

The polyphenols in tea leaves may be separated on a two-dimensional chromatogram with phenol and butanol–acetic acid (265).

Organic Acids

The lower fatty acids readily soluble in water, the hydroxy-acids and dicarboxylic acids are chromatographed either as free acids or as NH_4^+ salts. The higher fatty acids must be run as salts in butanol–ammonia, since the free acids are not sufficiently water-soluble. The fatty acids above C_{10} must either be separated as K-hydroxamates or on an inversed phase chromatogram as esters. Acidimetric indicators are used for developing.

R$_f$-*values of Organic Acids* (47)

	R_f-values in n-butanol saturated with $1{\cdot}5N$ NH_3
Formate	0·09
Acetate	0·10
Propionate	0·19
n-Butyrate	0·33
n-Valerate	0·45
n-Capronate	0·61
n-Caprylate	0·74
Benzoate	0·39
Salicylate	0·50
β-Phenylpropionate	0·52
Lactate	0·07
Oxalate, malate, partrate, adipinate, citrate	0·00
	R in benzene–acetic acid–water 4 : 4 : 2 (301)
p-Hydroxycoumaric acid	0·95
Salicylamide	0·9
m-Hydroxybenzoic acid	0·9
p-Hydroxybenzoic acid	0·9
p-Hydroxyphenylacetic acid	0·85
Salicyluric acid	0·75
2 : 4-Dihydroxybenzoic acid	0·7
m-Hydroxybenzamide	0·15
p-Hydroxybenzamide	0·10
m-Hydroxyhippuric acid	0·05
p-Hydroxyhippuric acid	0·05
2 : 5-Dihydroxybenzoic acid	0·5
2 : 5-Dihydroxybenzamide	0·25
Saligenin	0·95

1. Non-volatile acids readily soluble in water

E.g. malic, tartaric and citric acids, etc., are run with butanol–acetic acid the acetic acid then being evaporated off before spraying with indicator (202).

R$_f$-*values of Acids in Ethanol 80, conc. Ammonia 4, Water 16 on Whatman 54* (198)

Test: acidimetric indicator or specific reactions.

Acid	R_f	Acid	R_f	Acid	R_f
Formic	0·50	Arginine	0·20	Naphthalene-β-carboxylic acid	0·69
Acetic	0·52	Aspartic	0·18	*m*-Nitrocinnamic acid	0·83
Propionic	0·56	Cystine	0·10	Protocatechuic acid	0·39
Butyric	0·64	Glutamic	0·22	Salicylic acid	0·73
Valeric	0·65	Glycine	0·26	*o*-Toluic	0·63
Caproic	0·70	Lysine	0·60	*p*-Toluic	0·63
Caprylic	0·74	Phenylalanine	0·68	Oxalic	0·10
*iso*Butyric	0·64	Proline	0·44	Malonic	0·26
*iso*Valeric	0·67	Serine	0·32	Succinic	0·29
Phenylacetic	0·60	Threonine	0·40	Glutaric	0·32
Trifluoracetic	0·75	Tyrosine	0·48	Adipic	0·41
Chloracetic	0·52	Valine	0·61		

Acid	R_f	Acid	R_f	Acid	R_f
Dichloracetic	0·60	Carbobenzoxyphenyl-alanine	0·83	Pimelic	0·44
Trichloracetic	0·70	Benzoic	0·58	Maleic	0·31
Bromacetic	0·54	*o*-Bromobenzoic	0·76	Phthalic	0·39
β-Chlorpropionic	0·60	*p*-Bromobenzoic	0·76	*iso*Phthalic	0·45
Citric	0·11	Cinnamic	0·68	Terephthalic	0·45
Lactic	0·49	Naphthalene-α-carboxylic acid	0·69	Aconitic	0·16
Malic	0·25			Sulphanilic	0·53
Tartaric	0·19			*p*-Toluene sulphonic	0·73
Alanine	0·43				

2. The lower fatty acids (47, 48)

These are applied as sodium salts. As solvent *n*-butanol saturated with 1·5*N* ammonia of propanol 60, conc. ammonia 30, water 10 (336). The sodium ion does not migrate together with the fatty acid, but is exchanged with NH^-_4, and thus stays behind at the starting point. After running, the chromatogram is dried for 5 mins. at 95° and then sprayed with indicator.

Developer: (*a*) 40 mg. bromocresol green in 100 c.c. water or alcohol is treated with NaOH until the colour is just on the change to blue. Anions give yellow spots, cations deep blue spots on a pale blue background.

(*b*) Bromothymol blue of the same concentration as above. Brought up to p_H 10 with a few drops of NaOH. The reagent colours anions yellow and cations blue, on a green background.

(*c*) The following procedure may also be employed (156): The paper is sprayed with bromocresol green immediately after running. Initially everything turns blue, After standing for a few hours at room temperature, the ammonia volatilizes, and only the spots of fatty acids remain blue, since these are now present in the form of weakly alkyline soaps.

(*d*) Formic acid can also be detected with silver nitrate–ammonia.

α-Keto-acids form with *o*-phenylenediamine compounds which exhibit a strong fluorescence in uv-light, provided the paper has been previously treated with a strong acid. For the detection of really small quantities of α-keto-acid, the paper should be sprayed with a freshly prepared 0·05% solution of *o*-phenylenediamine in 10% aqueous trichloracetic acid and then dried for exactly 2 min. at 100° (329). After this treatment the paper should be slightly damp to the touch and should have assumed a pale yellow colour. The paper then fluoresces with a yellow-green colour in uv-light wherever

R_f-*values of α-Keto-acids* (203, 329)

	Propionic acid–water–*n*-butanol (5 : 7 : 10)	Water–α–*N*–dimethyl-amino*iso*butyronitrile–butanol (3 : 4 : 6)	Butanol–formic acid (95 : 5) sat. with water
Pyruvic acid	0·29	0·46	0·64
Dimethyl pyruvic acid	0·60	0·78	—
Cinnamoyl formic acid	0·75	0·95	—
α-Ketoglutaric acid	0·21	0·07	0·51
Oxalacetic acid	0·16	0·04	0·08
α-Ketobutyric acid	—	—	0·76
α-Keto*iso*valeric acid	—	—	0·85

α-keto-compounds are present. α-Keto-acids may also be recognized by t fact that their semicarbazones appear as dark shadows on a weakly fluoresce background in uv-light (203).

3. Higher fatty acids

The more easily soluble K-hydroxamates of the fatty acids C_1–C_8 may separated with *iso*butyric acid and phenol and then developed with fer chloride solution: red spots (113, 288). The very sparingly soluble high fatty acids are run as esters on an inverted phase chromatogram with pap which has been previously treated with caoutchouc latex (32, 33). T paper (Schleicher & Schuell 595) is treated with dilute vulcanized rubb latex, washed with alcohol and acetone and then stored under acetone. T paper may contain up to 50% caoutchouc without losing its porosity. So vents: methanol, acetone–methanol, benzene–methanol. It is also possib to work on a preparative scale in columns with caoutchouc powder. Anoth process utilizes silica gel which has been rendered lipophilic by treatment wi dichlorodimethyl-silane or paraffin (163).

Methanol containing 1% water may be used for running the higher fat acids, often on untreated paper (171).

Organic Bases

The chromatographic separation of organic bases offers no fundament difficulties. Thus, for example, choline, urea, spermine spermidine may readily differentiated (66, 318). The excretion of urea labelled with ^{14}C m be followed on the paper chromatogram (192). Aromatic amines are diaz tized on the paper after running and then coupled with *N*-ethyl-1-naphthy amine-HCl (104). After running, the chromatogram is dried and the sprayed with

1. a 0·2% nitrite solution in 0·1*N* HCl. After drying for a sho period at 50°, the paper is sprayed with
2. a 0·2% solution of ethyl-naphthylamine in alcohol.

R_f-*values of Organic Bases*

	Phenol–NH_3	Collidine	Butanol–acetic acid
Urea	0·73	0·44	0·43
Spermine	0·95	0·01	0·03
Spermidine	0·95	0·03	0·07
Adrenaline	—	—	0·31
Noradrenaline	—	—	0·25
Methyladrenaline	—	—	0·31

R_f-values of Aromatic Amines (104)

Solvent:					
% Methanol	40	35	35	35	30·8
% Amyl alcohol	20	17·5	17·5	17·5	15·2
% Benzene	20	35	35	35	46
% Water	20	12·5	12·5	12·5	8
			2*n* HCl	4% NH_3	
Sulphathiazole	0·86	0·78	0·53	0·64	0·64
Sulphanilamide	0·78	0·73	0·45	0·69	0·53
Sulphapyridine	0·87	0·82	0·53	0·73	0·72
Diaminodiphylsulphone	0·89	0·84	0·80	0·84	0·79
p-Aminobenzoic acid	0·88	0·56	0·79	0·41	0·71
o-Aminobenzoic acid	0·90	0·61	0·82	0·53	0·84
m-Aminobenzoic acid	0·81	0·42	0·78	0·45	0·76
p-Aminosalicylic acid	0·79	0·44	0·79	0·46	0·71
Sulphanilic acid	0·74	0·73	0·50	0·50	0·35
α-Naphthylamine	0·92	0·95	0·92	0·95	0·98
β-Naphthylamine	0·92	0·93	0·89	0·95	0·93
m-Phenylenediamine	0·62	0·75	0·35	0·71	0·50
p-Phenylenediamine	0·25	0·75	0·28	0·70	0·46
m-Toluidine	0·89	0·85	0·87	0·86	0·83
o-Toluidine	0·88	0·88	0·86	0·89	0·87
Aniline	0·88	—	0·71	0·91	0·84
p-Aminophenol	0·51	0·82	0·33	0·74	0·46

Adrenaline and noradrenaline are run in the form of their salts—i.e. in acid solvents—and exhibit a green fluorescence in an alkaline medium. The paper may also be sprayed with 0·44% potassium ferric cyanide solution in a buffer of p_H 8. The adrenaline then gives rise to rose-coloured spots, the noradrenaline to pale violet ones (108, 167, 282).

Diazotized sulphanilic acid may also be employed for developing. Adrenaline has an R_f-value of 0·32 in butanol–acetic acid, noradrenaline 0·25.

The amines listed in the following table (43) may all be developed with ninhydrin:

Amine	Butanol–acetic acid	Phenol–water sat.	Amine	Butanol–acetic acid	Phenol–water sat.
Methylamine	0·37	0·72	ββ-Diphenylethylamine	0·72	0·90
Ethylamine	0·45	0·80	β-Phenyl-β-hydroxyethyl-amine	0·65	0·86
n-Propylamine	0·58	0·86	Adrenaline	0·45	0·74
n-Butylamine	0·70	0·91	Agmatine	0·05	0·42
n-Amylamine	0·77	0·92	Allylamine	0·50	0·86
n-Heptylamine	0·85	0·94	Ethanolamine	0·33	0·65
iso-Propylamine	0·57	—	Dimethylamine	0·43	0·95
iso-Amylamine	0·77	—	Ephedrine	0·75	0·94
1 : 2-Diaminoethane	0·14	0·18	Glucosamine	0·24	0·30
1 : 3-Diaminopropane	0·15	0·25	Histamine	0·19	0·68
Putrescine	0·16	0·45	Spermine	0·07	0·24
Cadaverine	0·17	0·59	Tryptamine	0·67	0·91
1 : 6-Diaminohexane	0·20	0·67	Tyramine	0·62	0·85
Benzylamine	0·68	0·91			
β-Phenylethylamine	0·72	0·91			

Alkaloids may also be separated on the paper chromatogram. Th
curare alkaloids possess the following R_f-values (277):

	Org. phase from ethyl acetate 200, water 200, pyridine 90	Org. phase from methylethyl ketone 300, water 70, cellosolve 15
C-Curarine	0·24	0·42
C-Calebassine	0·27	0·37
C-Toxiferine I	0·19	0·20
C-Fluorocurine	0·53	0·59

These two solvents can also be employed for running a two-dimension chromatogram. Location by fluorescence or developing with ceric sulphat

Hyoscine and hyoscyamine (109), also atropine, various solanace alkaloids, brucine, morphine, strychnine, narcotine and the quinine alkaloi (65) are run with butanol on buffered paper and developed with dilut I_2/KI solution. The ergot alkaloids (65) are chromatographed with ethe which has been saturated with water, on paper buffered to p_H 2·6. Thes alkaloids can then be indentified in uv-light. Berberine, see (106). Add tional R_f-values of alkaloids see (229a).

The tobacco alkaloids should be run at 0° (on account of the considerab volatility of a number of these) in butanol–acetic acid. The chromatogra is then developed by submitting to the action of cyanogen bromide vapou for 10 min., subsequently spraying with a solution of 2% aniline and 0·25 benzidine in 50% alcohol (316, 182a).

R_f-*values of the Tobacco Alkaloids* (316)

Nicotine	0·45	Nicotinic acid amide	0·70
m-Nicotine	0·04	Aminobutylpyridine	0·52
nor-Nicotine	0·42	Anabasine	0·50
Nicotyrine	0·92	Anatabine	0·47

Quinoline esters (326) and imidazole derivatives (3) may be run as fre bases.

Sulphonamides may be diazotized and coupled on the paper as describe above. Diazotization in the gas phase also leads to good results (189): Th strip is suspended for 30 sec. in a cylinder, at the bottom of which a littl sulphuric acid has been poured on to some sodium nitrite. Alternativel Ehrlich's reagent may be employed for developing. The paper is spraye with a 1% solution of *p*-dimethylaminobenzaldehyde in dilute hydrochlor acid.

Ochre-coloured spots are obtained, which may then be extracted an estimated colorimetrically.

The following solvents are suitable: butanol 40, conc. ammonia 10, wate 50 (199); water-saturated butanol; see also (315).

Vitamins

Paper chromatograms of vitamins may be developed micro-biologically. A suitable-sized agar plate is uniformly inoculated with a bacterium strain which requires the vitamin in question for growth. The paper strip is then laid on top of the agar plate, which is then placed in an incubator. Bacterial growth now sets in wherever the growth factor was present. On raising up the paper, the agar is then found to have assumed a milky appearance at these points. If desired, the agar plate may also be photographed.

If the culture is subsequently sprayed with dilute glucose solution containing triphenyl-tetrazolium chloride, after a short incubation period formazane is set free by the vital activity of the bacteria at all points where the growth factor is present, thus giving rise to red spots on a colourless background.

By this method it is possible, for example, to detect 0·002γ vitamin B_2 (348). All water-soluble vitamins may be separated in this way.

The vitamins of the B_6 group—pyridoxal, pyridoxamine and pyridoxine—are developed with 'Saccharomyces carlsbergienis 4228'. The chromatogram is run with butanol, the solvent is evaporated, the paper is moistened and placed on a micro-biologically prepared agar plate. After incubation, the areas on which the vitamins are present become easily recognizable (340). The growth factors of the B_{12} group are chromatographed with *n*-butanol and developed with 'Lactobacillus Dorner' (81, 341, 349). The use of phenols as solvents is not permissible here because these act as inhibitors even in minute traces.

Vitamin A is chromatographed on paper which has been impregnated with Al_2O_3 (83).

R_f-*values of Vitamins* (20)

Phen = phenol saturated with an aq. soln. 6·3% Na citrate and 3·7% prim. phosphate.

Compound	Phen	Butanol–acetic acid	Developer	Colour
p-Aminobenzoic acid	0·84	0·86	$FeCl_3$	brown
Ascorbic acid	0·20	0·45	Silver NH_3	black
N-methyl-nicotinamide	—	0·84	UV	dark
Nicotinamide	—	0·77	Ferricyanide-nitroprusside	yellow
Nicotinic acid	0·95	0·79	Acid–indicator	—
Pantonine	0·83	0·43	Ninhydrin	violet
Pantothenic acid	—	0·81	Acid-indicator	—
Pyridoxine	0·32	0·73	$FeCl_3$	brown
Riboflavin	0·83	0·26	UV	yellow
Thiamine	0·25	0·36	Diazot. sulphanilic acid	orange

Chromatograms of plant growth factors may also be evaluated biologicall (200). The plant extracts are run with butanol–ammonia or *iso*propanol-ammonia. The chromatograms are afterwards cut into transverse strips o width 1 cm., each strip then being placed in a separate dish with 1 c.c water. After 17 hr. ten corn coeoptile slices of length exactly 2 mm. ar introduced into each dish and allowed to stand for 24 hr. at 25°. The lengtl of the slices in the individual dishes is then measured, the average lengtl being plotted on a graph. The location of the growth factors on the chro matogram may now be seen from the growth curve without difficulty.

β-Indolyl acetic acid exhibits an R_f-value of 0·35 in butanol–ammonia.

Inhibitors may also be chromatographed in this manner.

Antibiotics, Inhibitors

These may be treated in a manner similar to that used for growth factors Test bacteria are employed whose growth is inhibited by the antibiotic unde investigation. Clear areas then appear on the incubated plate wherever nc growth has occurred (129, 181, 342, 343, 344).

Subsequent spraying with TTC solution this time gives rise to white spots on a red background.

On account of the great sensitivity of the penicillins, these should be run at low temperature (4–5°) with water-saturated ether. The paper must be buffered to p_H 6·0 with phosphate buffer. 'Bac. subtilis' serves as a test bacterium. The penicillins G, F, dihydro-F and K may thus be separated and it is possible to establish which penicillin is produced preferentially by a particular mycelium. 'Staphylococcus aureus 209 P' was also used as a test bacterium.

Penicillin labelled with ^{35}S is developed autoradiographically (193).

The streptomycins are run with collidine/piperidine, lutidine/piperidine or butanol/piperidine containing 2% toluene sulphonic acid (water-saturated butanol 98, piperidine 2, *p*-toluene sulphonic acid 2 g.). In this case the paper should also be buffered to p_H 6–7. The bacterium employed is 'Staphylococcus aureus' or 'Bac. subtilis'. The streptomycins may also be developed with nitroprusside (286).

The following have been separated in this way: penicillins, streptomycin, mannosidostreptomycin, circulin, aureomycin, chloromycetin, dihydro-streptomycin, neomycin A, neomycin B, actinorubin (245, 259), the last named with 'Bac. coli' as test bacterium.

Porphyrins

Solvent: lutidine in an atmosphere saturated with lutidine and water vapour. Porphyrins exhibit a rose-coloured fluorescence in uv-light.

R_f-*values* (*232, 296*)

Uroporphyrin	0·07
Koproporphyrin	0·5
Protoporphyrin	0·75
Phylloerythrin	0·87

After pre-purification on a talcum chromatogram, the porphyrins of the urine may be investigated under normal and pathological conditions on an ordinary paper chromatogram (233).

R_f-*values in* 2 : 6-*Dimethylpyridine 50, Water 50 in an Atmosphere of Ammonia* (*174*)

Uroporphyrin	0·26	Mesoporphyrin	0·86
Koproporphyrin	0·54	Haematoporphyrin	0·87
Co-protoporphyrin	0·6	Deuteroporphyrin	0·88
Haeminporphyrin	0·7	Aetioporphyrin	1·0
Protoporphyrin	0·84	All esters	1·0

Sterols, Steroids, etc.

1. Solvents

As a consequence of the slight solubility in water of this class of compounds, it is not possible to run a chromatogram with them in the normal manner. An attempt was first made to convert them into water-soluble derivatives with the Girard T reagent, a procedure not found particularly useful owing to the minute quantities available (57). It is therefore best to avoid working with a distribution between water and an organic phase, employing instead a formamide/organic phase or propylene glycol/organic phase. Formamide and propylene glycol constitute very versatile solvents and are retained by the cellulose of the paper as the stationary phase (353). Benzene, toluene or, for glycosides, mixtures of these with chloroform are the usual solvents for the mobile phase. Only formamide may be used in the stationary phase with mixtures containing chloroform, because propylene glycol is readily soluble in chloroform. The speeds of migration are generally quite small, so that it is necessary to work with a continuous running chromatogram, running comparative substances simultaneously. Thus no

direct R_f-values are obtained. Should it be desired to separate several slow-running substances from one fast-running substance, it is necessary to run a number of test strips in order to ascertain just when the fast-running component reaches the end of the strip.

The paper is steeped in formamide, surplus solvent then being squeezed out between two sheets of filter-paper with a rubber roller. The benzene, toluene, etc., used in the mobile phase, must be saturated with formamide or propylene glycol respectively.

With reversed phases, the following method is employed (Hj. Staudinger, private communication): the paper (S & S 2043b) is moistened with water and allowed to dry. It is then impregnated with heptanol (15% in acetone). Ascending with water saturated with heptanol, the following R_f-values are obtained (developed with TTC):

Desoxycorticosterone	0·09
11-Desoxy-17-oxycorticosterone	0·15
Corticosterone	0·29
11-Dehydro-17-oxycorticosterone	0·45
17-Oxycorticosterone	0·45

The œstrogenes are coupled with diazotized *p*-nitro-benzene-azodimethoxyaniline and run with the organic phase from the following mixture: toluene 200, petrol ether 100, alcohol 30, water 70 (145, 146) (*v.* Table I, p. 93).

Another procedure employs a paper which has been rendered lipophilic with 'quilon'—Dupont (stearatochromyl chloride) (185). This is thus a reversed phase method, the cellulose forming mixed chromic acid–stearic acid esters with the reagent (*v.* Table II, p. 94).

Sterols may be chromatographed on Al_2O_3 paper (59). Whatman 54 is treated with a 20% solution of ammonium alum at 60° and then maintained for several hours in an atmosphere of ammonia. It is subsequently placed for 24 hr. in a 0·02% $CaCl_2$ solution and allowed to dry out in the air. A ready separation of e.g. progesterone ($R_f = 0{\cdot}85$), œsterone (0·4), and œstradiol (0·1) may be achieved with benzene. Œstrone, testosterone and androsterone should be separated with tetralin. Corticosterins are rather easily destroyed on aluminium oxide, but much more stable as 21-acetates.

The sexual hormones may be separated on silicon paper (see p. 100) (185a).

In general, the paper chromatographic separation with formamide as stationary phase is to be preferred.

2. Developing

The cortex hormones give black spots with alkaline silver nitrate solution and red ones with TTC.

Cortisone and others give blue spots on spraying with 0·3% I_2 in 5% KI solution.

The following method is more versatile (231):

The toluene/formamide chromatogram is dried and drawn through 15% phosphoric acid. Subsequent warming to 90° for 20 min. leads to the formation of fluorescent spots which appear in characteristic colour under the uv-lamp. The compounds are listed in Table III, p. 94, where they are arranged in descending order of increasing speed of migration—i.e. the ones at the bottom migrate most rapidly.

Glycosides: Spray with a, 10% *m*-dinitrobenzene and dry at 60°; b, 6 g. NaOH in 25 c.c. water and 45 c.c. methanol. Violet spots appear after 2 min., and begin to fade after 5–10 min. (262). The digitalis glycosides exhibit fluorescence after heating with trichloracetic acid (294). The hæmolytic action of the saponins may be utilized for their development (146a).

Table IV, p. 94, is intended to give a survey of the glycosides, showing their distances of migration in given times in cm. (262).

3. Quantitative estimation

The 17-hydroxy-sterones, being α-ketols, reduce tetrazolium salts so that it is possible to carry out a colorimetric estimation on this basis (58, 148, 156a).

The solutions obtained by extracting the spots on the chromatogram are evaporated in test-tubes to dryness *in vacuo*. The residue is subsequently treated with 0·2 c.c. of a 1% solution of 2 (*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride * and shaken until the steroid (about 5–50γ) has all passed into solution. One tube should be reserved for a blank test. The tubes are placed for 5–10 min. in a water-bath at 25° and then treated with 0·5 c.c. *N*/20 NaOH in 25% *iso*propanol, also pre-warmed to 25°. After 15 min. reduction is interrupted by the addition of 3 c.c. *N*/100 acetic acid in 90% *iso*propanol and the extinction coefficient at 480 mμ is determined. The relationship between the extinction coefficient and quantity is linear between 5 and 50γ. The extinction coefficients for 10γ in a tube 1 cm. long are:

Cortisone	0·13
17-Oxycorticosterone	0·14
Desoxycorticosterone	0·11
17-Oxydesoxycorticosterone	0·14

Table I. R$_f$-*values* (*145*)

Substance	Colour	R_f
Œstrone	purple	0·95
Œstradiol	,,	0·81
Œstriol	,,	0·07
Œstronsufate	,,	0·95
Equilin	,,	0·96
Phenol	red	0·50
Dehydro-corticosterone acetate	yellow	0·95
Progesterone	,,	0·74
cis-Testosterone	,,	0·21

* May & Baker, Ltd. Other tetrazolium salts also appear to be applicable (156a).

Table II (185)

R_f-value	Methanol	Ethanol	80% Ethanol
Cholestenone	0·77	0·97	0·86
Cholesterine	0·56	0·92	0·52

Table III (231)

Sterol	Fluorescence-colour	Limit of sensitivity
17-Hydroxycorticosterone	yellow-green	0·5 γ
Cortisone	blue	0·5–2
7-Hydroxycortexone	orange	0·5
Corticosterone	green	2–5
11-Dehydro-corticosterone	blue	25
Cortexone	violet	5

Table IV. Speed of Migration of Cardiac Glycosides in cm. Stationary Phase Formamide (262)

Benzene : Chloroform :	100% —	90% 10%	50% 50%	41·67% 58·33%	41·67% 58·33%	41·67% 58·33%	— 100%	
Time hrs. :	24	24	24	14	16	24	10	24
Strophanthidine	—	—	—	—	—	—	—	13·6
Sarverogenine	—	—	—	—	—	11·5	10·6	32·4
Sarmentogenine	—	—	—	—	—	—	—	8·0
Strophanthidol	—	—	—	—	—	—	—	14
Sarmentocymarine	4·1	7·5	25	—	19	22·2	D	D
Sarveroside	8·5	17	—	D	—	D	—	—
Cymarol	3·7	8	31	—	23	37	D	—
Cymarine	5·5	11	—	D	—	—	—	—
Emicymarine	1·5	1·5	—	3	4·5	—	—	20

D = substance ran completely through.

Synthetic Dyestuffs

It was in the field of synthetic dyestuffs that paper chromatography, in the form of the old process of capillary analysis, first found application. The results obtained using the new process with solvents containing water represent an essential improvement on capillary analysis (see coloured plates II and IV). It is, of course, only possible to achieve a separation of such dye-

stuffs as do not dye untreated cellulose (93, 228, 276, 284, 357). The following solvents are suitable: methanol, ethanol, butyl acetate, dioxane, ethyl acetate, tetrahydrofuran (276), butanol–water (93), and for the anthraquinone

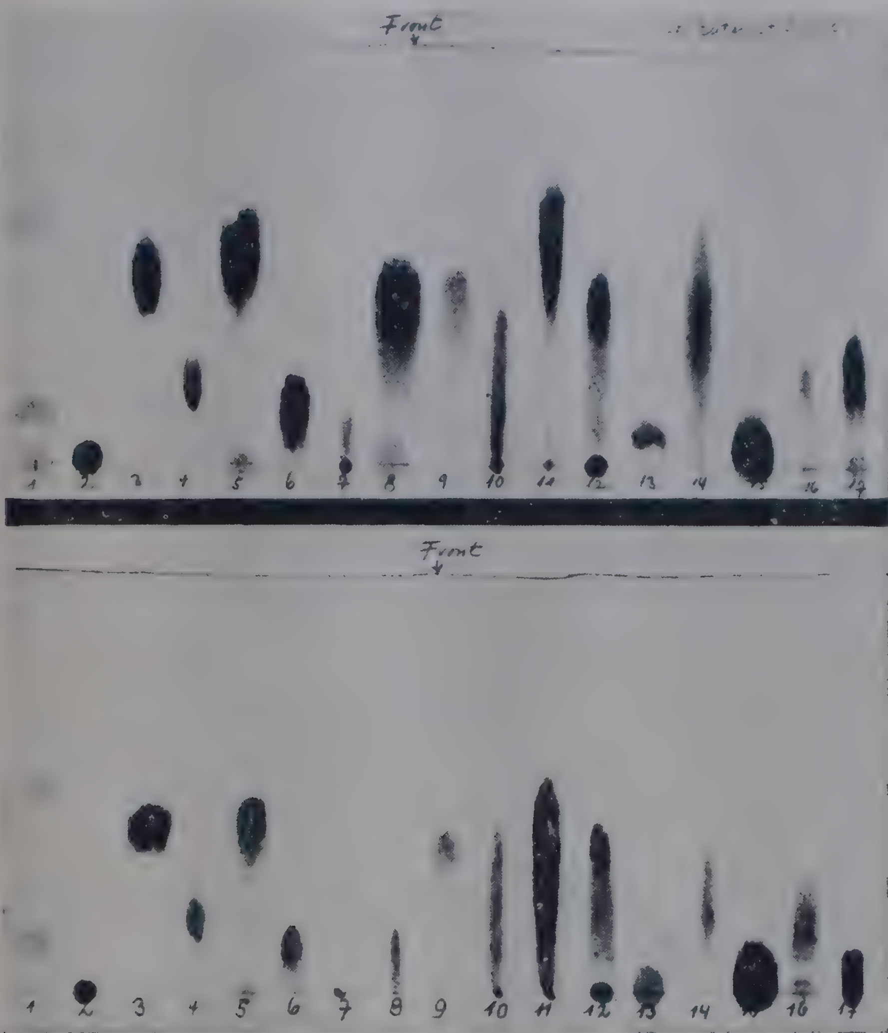


FIG. 66.—Paper chromatogram of wool dyestuffs on Schleicher and Schuell paper (357).

Above: *iso*butanol mixture.
Below: 80% *iso*butyric acid as solvent.

The numbers, 1–17, refer to the following table.

dyestuffs, petrol ether saturated with methanol at 19° (284). The following have proved especially useful (357):

*iso*Butyric acid 80, water 20
Glycol monoethyl ether 80, water 20
Ethanol 80, water 20
Glycol 60, water 40
*iso*Butanol 400, 2*N* acetic acid 75; 95 c.c. of this mixture treated with 5 c.c. Ethylene glycol (357).

Water alone seldom effects a satisfactory separation of mixtures of dyestuffs, since the spots are drawn out into long streaks. 80% alcohol suffices to separate a fair proportion of dyes into their components. An even better separation may be achieved, as illustrated below, by employing either an *iso*butanol mixture or *iso*butyric acid.

R_f-*values of Synthetic Dyestuffs* (357)

Paper Chromatography of Acid Wool Dyestuffs on Schleicher & Schuell 2043b Paper. (Ascending Method, Paper 30 × 15 cm., 15° C.)

Acid dyestuffs		R_f in 80% alcohol	R_f in *iso*butanol	R_f in *iso*butyric acid
1. Kitonlichtgelb 3 GRL *	yellow	0·51	0·00	0·00
	yellow	0·75	0·12	0·13
	yellow	0·86	0·57	0·49
2. Anthralanorgane GG	—	0·46	0·02	0·00
3. Orange II	—	0·85	0·45	0·41
4. Kitonlichtrot BGLE	—	0·73	0·19	0·18
5. Echtrot AV	violet	0·33	0·00	0·00
	red	0·75	0·48	0·41
6. Kristallponceau 6 R	—	0·65	0·14	0·10
7. Alizarinsaphirblau C	blue	0·0–0·34	0·00	0·00
	blue	0·58	0·10	—
8. Sulfocyanin 5 R extra	—	0·75	0·41	0·07
Mordant dyes				
9. Chromechtgelb O	—	0·72	0·41	0·34
10. Chromechtrot	—	0·57	0·25	0·24
11. Chromechtblau 2 RB	—	0·38–0·84	0·49	0·33
12. Säurealizarinschwarz R	—	—	0·62	—
	violet	0–0·64	0·39	0·36
	brown	0·72	—	—
	blue	0·94	0·57	0·49
	steel blue	—	0·00	0·00
Chromo dyes				
13. Neolangelb BE	—	0·54–0·86	0·08	0·02
14. Neolanrosa BE	—	0·00	—	—
	—	0·62	0·37	0·24
15. Neolanrot BRE	—	0·55	0·05	0·05
16. Neolangrün BF	—	0·00	0·27	0·16
	—	0·53	—	—
17. Neolanblau 2 RB	—	0·00	0·25	0·00
	—	0·53	—	0·08

* The three constituents of this dyestuff may readily be distinguished in uv-light.

Paper Chromatography of Wool Dyestuffs on Whatman 1 Paper (357). (Ascending Method, Paper 30 × 25 cm., 15° C.)

Acid dyestuffs	R_f-*iso*butyric acid 80%	R_f-ethyl alcohol 80%
2. Kitonechtgelb 3 GRL	0·05	0·62
	0·17	0·81
	0·65	0·95
3. Orange II	0·55	0·89
4. Kristallponceau 6 R extra	0·18	0·75
5. Echtrot AV	0·00 (traces)	0·28
	0·54	0·91
6. Kitonlichtrot BGLE	0·19	0·85
7. Alizarinsaphirblau	0·00	0·29
	—	0·73
8. Sulfocyanin 5 R extra	0·19	0·96

Acid dyestuffs	R_f-*iso*butyric acid 80%	R_f-ethyl alcohol 80%
Mordant dyes		
Chromechtgelb O	0·24	—
Chromechtrot O	0·36	—
Chromechtbraun 6 GBL	0·72	—
Chromechtblau 2 RB	0·34	—
Säurealizarinschwarz R	0·00	—
	0·24 (violet-red)	—
	0·54 (blue-green)	—
Chromo dyes		
Neolangelb BE	0·01	—
Neolanrosa BE	0·08	—
Neolanrot BRE	0·007	—
Neolangrün BF	0·10	—
Neolanblau 2 G	0·023	—

Separation of Optical Isomers (34, 183)

For the separation of optical isomers on the paper chromatogram, it is necessary to employ optically active solvents. In this way acidic substances (183) may be resolved using 1-methyl-(β-phenyl*iso*propyl)-amine as basic solvent and racemic bases can be cleaved with solvents containing *d*-tartaric acid (34). The paper is first impregnated with phenol, after which the base is applied and then run with *d*-tartaric acid. In a trial run with β-naphthol-benzylamine the *l*-compound was found to have an R_f-value of 0·18 and the *d*-compound 0·47.

Inactive solvents may, however, also be employed to separate optical isomers on the paper (81a).

Various tryptophane and phenylalanine derivatives may be resolved with butanol–acetic acid. The ratio of the R_f-values of the D and L forms is 0·9. Only a small number of α-amino-acids respond to this treatment. If the amino-group is substituted, resolution is no longer possible. The process is explicable only on the assumption that the optical activity of the cellulose is responsible for the separation. This means that adsorption effects also play an essential rôle in ordinary paper chromatography. Whatman No. 4 was employed in these experiments.

Chromatography on Pre-treated Paper, Reversed-phase Chromatography

1. Carboxyl paper (338)

By cautious oxidation of cellulose, it is possible to convert the hydroxy-groups on C_6 to carboxyl groups. If oxidation is carried to a stage beyond 5% COOH, the cellulose is rendered alkali-soluble. For a carboxyl content

FIG. 67.—Paper chromatogram of various amino-acids on paper of 1% carboxyl content.

Solvent: ammonium formate solution (m/20, p_H 9) (338).

FIG. 68.—Paper chromatogram of a selection of cations on paper of 1% carboxyl content.

Solvent: ammonium acetate solution (m/10, p_H 3). Developed with H_2S (338).

between 1% and 4% the cellulose retains its external properties, but is, nevertheless, able to function as an ion exchanger.

Amino-acids. With water as solvent, the basic ones remain behind at the starting point, since they form salts with the carboxyl groups. The others migrate with the front. In a buffer at p_H 8 the basic ones migrate with varying speeds.

Amines and alkaloids may be readily separated. The R_f-values in the same solvent are 0·45 for cysteamine and 0·16 for histamine.

Inorganic ions may be run with $M/10$ ammonium acetate. The damp chromatogram is developed by exposing to H_2S. At this buffer concentration, silver does not separate well from lead, a satisfactory separation of

which, however, may be achieved by employing an $M/20$ buffer at the same pH, when the lead remains back at the starting point. Separation may thus be accomplished on a two-dimensional chromatogram. Separation of arsenic, antimony and tin also presents no difficulty at pH 5 with $M/20$ ammonium acetate. The antimony then remains as antimonyl ion near the starting point, while the arsenic migrates as arsenate with an $R_f = 0{\cdot}8$ and bivalent tin runs with the front.

In contradistinction to the usual ion exchange chromatography, this process offers considerable advantages. Thus it is possible to work with the minutest quantities; developing is easy and results are clear. For work on a preparative scale, powder of the same material may be employed.

2. Aluminium oxide paper

A strip of hard paper (Whatman 54) is first plunged into a solution of aluminium sulphate (65 g/l.) and then into a bath of $2N$ ammonia (83). If it is desired to impregnate the paper more strongly, a more concentrated aluminium sulphate paper may be employed, the strips afterwards being suspended in an atmosphere of ammonia. This paper may then be used for separating vitamin A alcohol from its ester using petrol ether, b.p. 80–100°, as solvent. A paper of this type functions just like a column of aluminium oxide. The carotins, however, may also be run on untreated paper (15, 171).

The separation of the sterols is discussed elsewhere (p. 92).

3. Silica gel paper

In order to separate homologous aldehydes, these are converted into the respective 2 : 4-dinitrophenylhydrazones and chromatographed. The paper is pre-treated with a solution of water-glass followed by HCl, washing and drying at 110°. Solvent: 5% ether in petrol ether (110°) (179).

R_f-*values of Dinitrophenylhydrazones*

	Untreated paper	Pre-treated paper
Methylethyl ketone	0·90	0·38
Methylpropyl ketone . . .	0·90	0·54
Methyl *iso*propyl ketone . . .	0·91	0·48

4. Chromatography with reversed phases

The polar, hydrophilic phase on a normal paper chromatogram is on the cellulose. It is therefore only possible to run such substances as are at least to a certain extent soluble in water; the others run with the front. In

order to extend the applicability of paper chromatography to lipophili substances, various attempts have been made to render the cellulose of th paper lipophilic. Polar liquids like alcohol, aqueous alcohols, etc., are the employed as solvents—i.e. mobile phase.

Paper strips are placed in a dilute solution of vulcanized caoutchouc late and then removed and dried. The paper should then contain 30% caout chouc. The strips are washed in alcohol and acetone and stored unde acetone until required. It was found possible to separate esters of the highe fatty acids with benzene–methanol (32, 33). See also under organic acids.

The gammexanes (hexachloro*cyclo*hexanes) may be separated on pape which has been previously steeped in acetic anhydride. The five isomer may then be run with *n*-hexane or petrol ether, b.p. 60–80° (226). On com plete acetylation of the paper with acetic anhydride–sulphuric acid in benzene the structure of the paper, although now rendered lipophilic, remains essen tially unchanged and it is thus possible to carry out separations with water insoluble substances (36, 215a).

The paper is saturated with a 2·5% ethereal solution of vaseline an allowed to dry. DDT-derivatives may then be run on this paper wit ethanol 80, water 15, conc. ammonia 5 (345). The various tocophero derivatives may also be separated on the same paper with 75% ethanol (49)

Treatment of the paper with silicones also presents no difficulties (185a) The paper strips are dipped into a 5% solution of 'Dow Corning Silicone No 1107' in *cyclo*hexane, pressed out and dried for 1 hr. at 110°. The paper i now hydrophobic and capable of adsorbing organic solvents. This treat ment is irreversible. A suitable solvent is water, abs. alcohol, chloroforn 6 : 10 : 10. The lower phase is employed for saturating the atmosphere i the cabinet, the upper more strongly polar one, for running the chromato gram. This procedure has hitherto found application in the separation o the sexual hormones.

Inorganic Paper Chromatography

The use of organic solvents in inorganic analytical chemistry was hitherto limited to a small group of elements, as, for example, in the separation o Au–Pt, K–Na, $SrCl_2$–$BaCl_2$, $Ba(NO_3)_2$–$Ca(NO_3)_2$ or Zr–Hf. The pape chromatographic separation of inorganic ions, however, provides proo enough that, in fact, a large number of inorganic salts possess sufficient, an often considerable, solubility, so that migration on the paper takes place. A a result of differing distribution coefficients between two phases, numerou fractional separations are then possible (see coloured plate III).

It is well know that cellulose can function as an ion exchanger, so that this separation is not solely due to a simple distribution between two phases. For all practical purposes of inorganic paper chromatography, however, this whole question of mechanism is, of course, immaterial; numerous elements, including closely related ones, may be separated in a rapid and elegant manner on the paper chromatogram (7, 8, 16, 53, 55, 190, 191, 235, 249).

1–100γ of the individual elements may be separated, or quantities up to 1 mg. using a strip chromatogram. The metals are generally applied to the paper in the form of salts, usually the chlorides. It should be borne in mind that hydrolysis often sets in on drying at higher temperatures. The drying period may thus often be of considerable importance. The distance from the rim of the trough to the starting line should also be kept as constant as possible.

Inorganic paper chromatography is carried out on short strips up to 30 cm. in length. The trough and cabinet employed should not be much larger than necessary for the size of chromatogram being run, otherwise too much time elapses before the inside atmosphere has become saturated with solvent vapours.

The solvents employed should be freshly distilled and of analytical purity, since complex forming impurities can give an entirely false picture.

The use of the following solvents for the separation of the cations, applied as nitrates, represents a universally applicable method (250, 251):

1. Butanol 50, 0·1*N* hydrochloric acid 50, benzoyl acetone 0·5. The cations indicated separate with the following R_f-values:

Ag	0·10	Cu^{2+}	0·22	Sn^{2+}	0·58
Hg^{+}	0·24	Cd^{2+}	0·05	Sn^{4+}	0·55
Hg^{2+}	0·31	As^{3+}	0·43	Fe^{3+}	0·95

2. Collidine 50, 0·4*N* nitric acid 50.

Ag^{+}	0·78	Zn^{2+}	0·75	Sr^{2+}	0·40
Cu^{2+}	0·76	Mn^{2+}	0·71	Mg^{2+}	0·65
Cd^{2+}	0·76	Co^{2+}	0·74	K^{+}	0·32
As^{3+}	0·65	Ni^{2+}	0·76	Na^{+}	0·42
Sb^{3+}	0·38	Ca^{2+}	0·52		

3. 1 c.c. conc. nitric acid and 2·5 c.c. water are added to 100 c.c. dioxane containing 1 g. dissolved antipyrine.

Ag^{+}	0·08	Bi^{3+}	0·63	As^{3+}	0·18
Hg^{+}	0·43	Cu^{2+}	0·24	Sn^{2+}	0·77
Pb^{2+}	0·15	Cd^{2+}	0·18	Sn^{4+}	0·58

A solution of kojic acid and 8-hydroxyquinoline (0·1 g. kojic acid + 0·5 g. 8-hydroxyquinoline in 100 c.c. 60% ethanol) is a universal developer of extremely wide application. The paper is sprayed and exposed to ammonia, after which characteristic fluorescent spots or dark patches become visible in uv-light.

Generally, however, it is only necessary to separate the elements within

some definite group. Numerous processes have been worked out fo particular groups of elements. Cations are distinguished by their R_f-value and characteristic colour reactions.

Separation of Pb, Cu, Bi, Cd, Hg (55)

As chloride: solvent, *n*-butanol saturated with $3N$ HCl. The acid chloride solution is applied and dried. Time of running, 15–18 hr. Dr Sprayed with dithiozone in chloroform, for lead with aqueous rhodizon acid.

	R_f-value	Reagent	Colour
Cu	0·20	Dithizone	purple-brown
Pb	0·27	Rhodizonic acid	blue
Bi	0·59	Dithizone	purple
Cd	0·77	,,	,,
Hg	0·81	,,	rose

H_2S may, of course, also be used for developing, but the reagents cite yield results which are more characteristic.

This group may also be run with other solvents; in the absence of lea dry pyridine is employed. The quantities of each metal applied are of th same order as used when working with organic substances.

As, Sb, Sn (55)

Solvent: 75 c.c. acetyl acetone saturated with water and treated wit 0·5 c.c. HCl (s.g. 1·18) and 25 c.c. acetone so as to clarify the solution. Th elements are applied as chlorides of the lower valency in hydrochloric aci solution. These cations run about 15 cm. per hour—Sn forms a comple

As	0·2	yellow
Sb	0·5	reddish
Sn	1·0	purple

with acetyl acetone so that it migrates with the solvent front. The incom pletely dry paper is sprayed with dithizone. The spots require som considerable time before reaching full intensity.

Fe, Al, Cr (55)

These are applied as trivalent chloride in $5N$ HCl and then allowed to d in the air. Solvent: glacial acetic acid 75, methanol 25 in a damp atmospher Developing: The strip is cut down the middle. One half is sprayed wit alizarin, fumed with ammonia and heated. Al red, Fe purple. The oth half is first sprayed with an aqueous solution of Na_2O_2 followed by benzidine glacial acetic acid. Chromium appears as a blue band.

Order: Al (solvent front), Cr, Fe.

Ni, Mn, Co, Zn (55)

These metals are run as chlorides. Solvent: acetone containing 5% water and 8% hydrochloric acid (s.g. 1·18). Dried, fumed with NH_3,

R_f-*values*

Ni	0·07	blue
Mn	0·3	brown
Co	0·6	brown
Zn	0·09	purple

developed with an alcoholic solution of alizarin, thiocyanic acid and salicylaldoxine.

Ni and Co may also be separated with acetone, alcohol and hydrochloric acid (188).

Ca, Sr, Ba (55)

These metals should also be present as chlorides.

Solvent: pyridine containing 20 vol. % water and 1% KCNS.

The atmosphere in the cylinder should be about 70% saturated with water vapour, which can be achieved by placing a small vessel containing

R_f-*values*

Ca	0·95
Sr	0·75
Ba	0·15

saturated ammonium chloride solution at the bottom.

Alizarin is used for developing Ca, Na rhodizonate for Sr and Ba.

Li, Na, K (55)

are chromatographed as chlorides in neutral solution with methanol. A

R_f-*values*

Li	0·8
Na	0·5
K	0·1

solution of $AgNO_3$ and fluorescein is employed for developing.

Alkali and alkaline-earth metals (107)

These may all be separated in one operation. They are applied as acetate in acetic acid solution and then run with ethanol 80, $2N$ acetic acid 20 on an ascending chromatogram.

Developing: spray on aqueous $0{\cdot}1M$ violuric acid and develop at 60°.

Lithium and magnesium have the same R_f-value, but may be distinguished by their colour. A semi-quantitative determination according to the spot size is possible here (280).

Metal	R_f-value	Colour
Li	0·76	reddish-violet
Na	0·56	,,
K	0·45	violet
Be	0·86	yellowish-green
Mg	0·76	yellowish-rose
Ca	0·68	orange
Sr	0·55	reddish-violet
Ba	0·43	bright red

Pt, Pd, Rh, Ir, Ru, Os, Au (55)

These metals should be present as chloride or as the sodium salt of th complex chloro-acid.

Solvent: methyl ethyl ketone 70, HCl (s.g. 1·18) 30, or methyl prop ketone. The solutions decompose fairly rapidly.

Developing Pt, Pd, Au, Rh are sprayed with stannous chloride or $SnCl_2$ - KI. For Rh, gentle heating is necessary. Ir is oxidized to the brown tetra chloride with chlorine water. Ru and Os are sprayed with a solution o thiourea in 5*N* HCl and heated.

R_f-*values*

Au	0·95	Pd	0·60
Os	0·91	Rh and Ir	0·10
Pt	0·80	Ru	0·07

Ir migrates as the trivalent ion (in the strongly acidic mixture, it is, i any case, present in this valency state), together with rhodium. As tetra valent ion, it migrates along with platinum. In order to separate rhodiur from iridium it is necessary to employ a second solvent. Now in aceton containing 5% HCl (s.g. 1·18) Ir^{4+} migrates with the solvent front, wherea rhodium remains stationary. The strip is therefore dried after the first run oxidized with chlorine water, dried once again and then run for a short tim with acetone hydrochloric acid until the acetone has traversed about a quarte of the strip. Iridium, if present, will then be located at the new front, be tween rhodium and palladium.

Separation of Au from the platinum metals (55)

The metals should be applied as chlorides in 2–3*N* hydrochloric acid an then dried for a short period.

Solvent: ether containing 1–2% dry HCl and 7·5% methanol. Thi separation is based on the fact that chlorauric acid is readily soluble in ether In this manner it is possible to detect 1γ gold in the presence of more thar 100γ platinum.

Ag, Cu, Pd, Pt, Au (190)

are separated with *n*-butanol saturated with 1*N* HCl.

Separation of Hg from the other metals (55)

Solvent: methyl acetate 87, methanol 3, water 10.

$HgCl_2$ is especially soluble in organic solvents and migrates in the above mixture with the solvent front, while Pb, Cu, Bi, Cd, As, Sb, Fe, Al, Cr, Ni, Co, Mn, Zn all remain back at the starting point.

Se, Te (55)

These elements should be present as selenite and tellurite in dilute HNO_3. Solvent: butanol 60, methanol 40. The necessary relative humidity of 50% is achieved by placing a saturated solution of calcium nitrate in the cabinet. The chromatogram is developed with $SnCl_2$ solution.

Te (R_f 0·1) black, Se (R_f 0·5) orange.

By using a strip which tapers down to a point, it is possible to detect 1γ selenium in the presence of 1 mg. tellurium.

Th, Sc, rare earths (55)

These metals are run as nitrate. Solvent: tetrahydrasylvane 85, water 5, HNO_3 (s.g. 1·42) 10. A humidity of about 80% is necessary. Developing: fumed for 10 min. with NN_3, sprayed with alcoholic alizarin solution, followed by 1*N* acetic acid.

R_f-*values*

Th	0·04	violet
Sc	0·17	,,
Rare earth	0·00	,,

1γ scandium detectable in 1 mg. rare earths.

F, Cl, Br, I (55)

The halogens are chromatographed as sodium salts. Solvent: pyridine 90, water 10.

Developing:

1. Cl, Br, I. Spraying with a solution of $AgNO_3$ and fluorescein gives rise to black spots which, under uv-light, also appear as black patches on a fluorescent background.

2. F. The other half of the divided strip is sprayed with a zirconium–alizarin solution.

R_f-*values*

F	0·00	Br	0·47
Cl	0·24	I	0·71

Al, Be (235)

Solvent: butanol 80, conc. HCl 20. Developed with an alcoholic solution of 8-hydroxyquinoline. Fluorescent spots are obtained which intensify on treating with NH_3. Amounts ranging from 2 to 300 may be separated.

R_f-values

Al . . .	0·03	Be . . .	0·30

Ni, Cu (95)

These metals possess different speeds of migration in an electric field anc may thus be separated by paper electrophoresis. Ni migrates approximatel twice as fast as Cu.

Al, Ga, In, Zn (55)

These metals are applied as chloride in acid solution. Solvent: water saturated butanol containing 10% conc. HCl. Relative humidity 65% Ga and In are sandwiched between Al, which fails to migrate at all, and Zn which runs with the solvent front. Al and Ga are developed with alizarin In and Zn with dithizone.

Fe, Co, Ni, Mn, Cu, Zn (55)

These are run as chlorides in methyl ethyl ketone 92, conc. HCl 8. R_f values are given in the table.

Ni, Co and Cu are developed with thiocyanic acid, Mn with ammoniaca silver nitrate and Fe with potassium ferrocyanide.

U (55)

Run as nitrate. Solvent: tetrahydrofuran 95, nitric acid 5 (s.g. 1·42) U runs with the front, all other metals remain behind. Developer potassium ferrocyanide.

A selection of R_f-values is appended in the following table:

Metal	Methyl ethyl ketone 92 HCl (d = 1·18) 8	Butanol saturated with $3n$-HCl
Ni	0·1	—
Mn	0·18	—
Co	0·54	—
Cu	0·71	0·19
Fe	0·93	0·42
V	0·14	0·18
U	0·87	0·26
Pb	—	0·23
Mo	—	0·53
Bi	—	0·57
Sb	—	0·78

Anions (250)

are chromatographed with butanol 40, pyridine 20, 1·5 ammonia 40.

Anion	Butanol/Py	Anion	Butanol/Py	Anion	Butanol/Py
Cl^+	0·24	JO_3^+	0·09	CO_3^{2+}	0·06
Br^+	0·36	NO_2^+	0·25	PO_4^{3+}	0·04
J^+	0·47	NO_3^+	0·40	CrO_4^{2+}	0·00
ClO_3^+	0·42	AsO_3^{3+}	0·19	CNS^+	0·36
BrO_3^+	0·25	AsO^{4+}	0·05	SO_4^{2+}	0·07

Circular filter chromatography of inorganic ions also yields good results (307a), especially as it is then possible to develop in sectors.

Two-dimensional chromatography (105)

For two-dimensional separation of cations the chromatogram is run one way with collidine and the other with butanol saturated with 0·1*N* nitric acid and 0·5% benzoyl acetone. The latter forms complexes with a number of metals, which then migrate more rapidly. Most metals can be developed with H_2S or 8-hydroxyquinoline–kojic acid (fluorescence).

It is also possible to employ filter-paper impregnated with aluminium hydroxide and dried. Water is then employed as solvent just as in an aluminium oxide column (119).

Quantitative Inorganic Paper Chromatography

An estimation of the amounts applied is possible from the size of the spots (8, 16). More accurate results are, of course, to be obtained by extracting the spots, subsequently carrying out a polarographic or other micro-determination.

On a preparative scale it is possible to separate inorganic salts on cellulose columns (54). For this purpose it is of advantage to reflux the cellulose or paper used for 2 min. with 5% nitric acid, subsequently washing with water, alcohol and ether. This treatment activates and oxidizes the surface layers of the cellulose, thus rendering it more suitable for the separation of cations, which incidentally provides an indication that adsorption and ion exchange play an important part in the process. In order to prevent surface effects at the wall of the column, the glass is treated with dichlorodimethylsilane for a short period previous to filling.

The solvents are generally the same as in the qualitative method.

The fluorides of niobium and tantalum may be separated with water-saturated methyl ethyl ketone (56). Ta first of all runs straight through the column, after which 7·5% HF (40% aqueous solution) is added to the solvent, which then runs the Nb. The separation is quantitative.

This doubtless represents a very promising method for the solution of a number of interesting problems—e.g. for the separation of Zr–Hf—but it remains to be perfected in its details.

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